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THE ACTION OF SULPHATED POLYSACCHARIDES UPON SERUM LIPIDS UNDER CLINICAL AND EXPERIMENTAL CONDITIONS

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APART from its anticoagulant activity, the property of heparin which has been most widely studied is its " clearing " action upon lipaemic plasma (Hahn, 1943). This action, which is due to activation of a lipase, is readily obtained in alimentary lipaemias and experimental conditions. It was found however that it was much less readily demonstrable in patients with abnormal lipaemias and that other anticoagulants possessed little or no lipolytic power. The activity of heparin was therefore examined afresh on the lines described below.

METHODS

Rabbits (adult males of various breeds) were made lipaemic by feeding continuously with diets containing enough cholesterol to give a daily intake of 0·5–1·0 g. The cholesterol was dissolved in petroleum ether which was lost by evaporation after addition to the standard MRC Diet 18. The final recoverable cholesterol content of the diet was 0·84–1·04 g. per 100 g. and the natural phospholipid content about 400 mg. per 100 g. of dry pellets.

Rats (young males, 110–150 g.) were made hypercholesterolaemic by feeding with a basic standard MRC Diet 41 (Bruce and Parkes, 1949) containing 0·5 per cent cholesterol and 0·1 per cent thiouracil w/w (Fillios, Andrews, Mann and Stare, 1956) for five weeks.

Cholesterol was extracted from serum or plasma and estimated by the Liebermann-Burchard reaction described by King (1951), the absorptions through an Ilford filter at 680 mμ being read at maxima.

Phospholipids were estimated by a modification of the method of Zilversmitt and Davis (1950) using trichloracetic acid precipitation and stannous chloride reduction.

Phenol-reactive lipids were estimated by the turbidity technique of Kunkel, Ahrens and Eisenmenger (1948).

Optical densities of untreated sera were read in a Spekker absorptiometer, using a 0·25 cm. cell and a 680 mμ filter.

Electrophoresis was performed in a vertical Flynn and de Mayo tank in a barbiturate buffer of pH 8·6. Serum or plasma was applied in 0·07–0·14 ml. volumes to 5 cm. Whatman No. 1 strips through which 0·2–0·25 mA/cm. was applied for 16 hours in a current-stabilised circuit. Effective separation of lipoprotein complexes was not obtained unless the albumin

zone travelled at least 10 cm. The strips were split longitudinally into three, one length being stained by azocarmine for protein, the second by sudan black for lipid and the third retained for cholesterol zone-detection. The α-and β-lipoproteins were plotted from the (broad) sudan-stained strip by photometric scanning and the quantities calculated as percentages of total stainable lipid, the zone boundaries being set in all strips from the azocarmine-stained replicate. The cholesterol zone was detected in the (narrow) third strip by applying Lieberman-Burchard reagents and marking the zone before disintegration of the paper on the other strips.

RESULTS

Experiments upon rabbits.—Inclusion of 0·5–1·0 g. cholesterol daily in the diet of adult rabbits raised the serum cholesterol from the normal value of 50–70 mg. per cent to about 500 mg. per cent in a month and to 800–1300 mg. per cent in 6–8 weeks, at which point experiments were started. Other types of lipid also showed an increase, and considerable quantities of various lipid fractions, including sudanophilic neutral fat, were deposited in the liver, kidney and other tissues (Table I). The classical atheromatous deposits in the aorta were conspicuous in animals killed at this stage. It is well known that the addition of cholesterol to the diet of the rabbit—a natural herbivore—produces a highly artificial hypercholesterolaemia ; the increase in the other lipid fractions has been studied by Popjak (1946) but the other pathological features of this interesting lipoidosis have attracted much less comment in the literature.

TABLE I.—*Lipid Fractions in Various Tissues of Cholesterol-fed Rabbits*

		Controls (4)	Cholesterol-fed (7)
Cholesterol	Serum mg./100 ml.	44–72	438–1280
	Aorta mg./100 g.	210–320	505–1300
	Liver mg./100 g.	210/320	2080–2350
Phospholipid	Serum mg./100 ml.	87–140	195–575
	Aorta mg./100 g.	250–290	390–1170
	Liver mg./100 g.	250–290	1900–2080
Fatty acids	Serum mg./100 ml.	93–154	179–740
Total phenol-reactive lipids	Serum mg./100 ml.	288–342	438–1694

The bulk of the abnormal lipid in the blood was always cholesterol, but there was a proportionate rise in phospholipid and in the whole lipoprotein complex, which was mainly of the slow-moving β-type on electrophoretic zone analysis (Fig. 1).

Heparin (500–1000 units per kg.) was injected into 3 normal and 6 lipaemic rabbits. Serum or plasma, sampled before and 3–20 min. after injection, showed increased optical densities when kept at 37° and usually became turbid. This occurred in normal and lipaemic animals but was much more marked in the latter, especially when the plasma was kept at room temperature or 4°, when the optical density often increased up to four times the pre-injection reading (Fig. 2). The turbidity was due mainly to the appearance of particulate fat, easily visible under dark-ground microscopy and not prevented or abolished by the presence of albumin or an excess of citrate. In very lipaemic plasma, a film of fatty droplets sometimes separated and centrifugation caused a layer of parti-

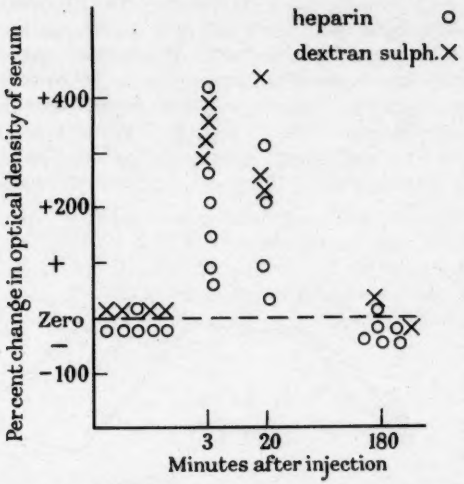

FIG. 2.—Effect of injections of heparin and dextran sulphate (1000 units/kg.) upon the optical density of the serum in rabbits with hyperlipaemia.

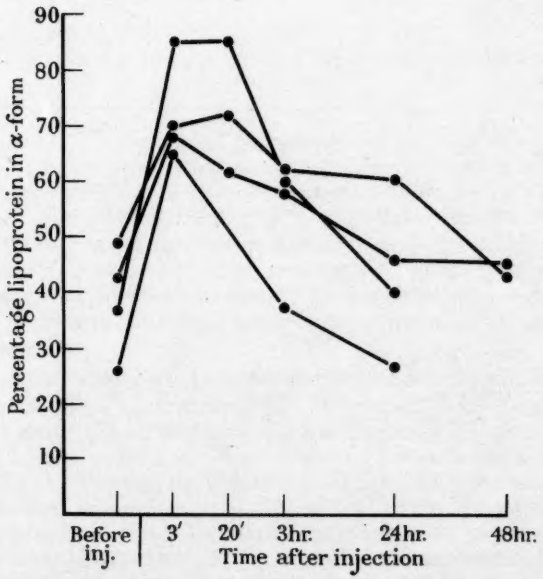

FIG. 3.—Changes in proportion of alpha-type lipoprotein in hyperlipaemic rabbits following injections of heparin (1000 units/kg.).

culate fat to rise. At the same time, phenol-reactive lipids increased (Table II), but not total extractible lipid, and there was no consistent alteration in cholesterol or phospholipids. A change was also found in plasma examined electrophoretically, when it was found that much of the slow-moving β-lipoprotein, characteristic of the lipaemic rabbit, changed under the influence of heparin to faster-moving α-components (Fig. 1 and 3). These changes began to revert to the normal within 3 hr. and some " clearing " of the plasma occurred. Some difference in the α-β distribution of lipoprotein persisted beyond this.

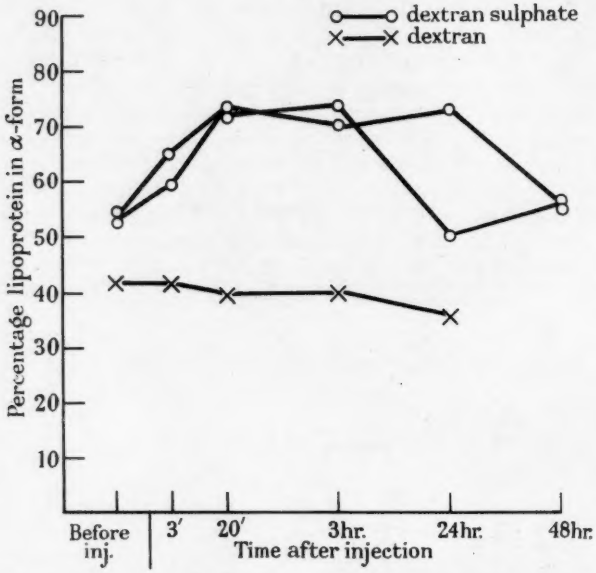

FIG. 4.—Changes in proportion of alpha-type lipoprotein in hyperlipaemic rabbits following injections of dextran sulphate (1000 units/kg.) and dextran (20 mg./kg.).

TABLE II.—*Lipid Fractions of Plasma in Lipaemic Rabbits Before and After Injections of Heparin* (500–1000 *units/kg.*)

Rabbit	Optical density (Extinction at 680 mμ)	Phenol-reactive (g.%)	Cholesterol (g.%)	Phospholipids (g.%)
1	0·25–0·60	3·1–3·9	3·1–1·8	0·9–0·6
2	0·29–1·32	3·9–4·5	2·7–2·1	1·0–0·8
3	0·22–0·28	2·0–2·5	1·7–1·7	—
4	0·27–0·32	2·8–2·8	2·0–1·7	—

Dextran sulphate (1000 units/kg.) produced similar but more lasting changes when injected intravenously. Reversion to the normal did not begin until after 3 hr. (Fig. 4). When dextran (not sulphated) was injected (20 mg./kg.) no changes were observed in optical density, total lipid or lipoprotein fractions.

Protamine sulphate was injected intravenously, before and after heparin or dextran sulphate, in doses calculated as their equivalents biologically (by reversal of the anticoagulant effect). The increase in optical density of the plasma and the separation of particulate fat were not prevented or abolished by protamine, though some incomplete neutralisation of the α-β lipoprotein interconversion was noted.

Experiments in rats

After 5 weeks on the thiouracil-cholesterol dietary régime described above, the serum cholesterol levels in 4 from a batch of 6 rats were 232–570 mg. per cent compared with original levels for the batch of 60–100 mg. per cent. Their lipoprotein, in contrast to that in the hyperlipaemic rabbit, was almost entirely of the fast-moving α-type, its position electrophoretically corresponding to that

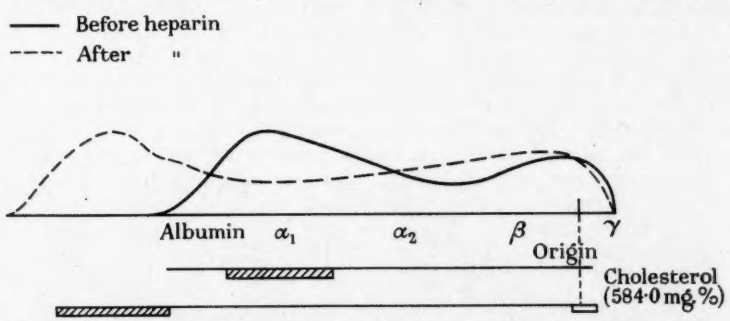

FIG. 5.—Effect of heparin (1000 units/kg.) upon the electrophoretic pattern of the serum lipoproteins in a rat.

of human α_1-globulin. Injections of heparin or dextran sulphate (1000 units/kg.) into the tail vein caused this α-lipoprotein complex to become more mobile and travel electrophoretically faster than the plasma albumin (Fig. 5). No change in cholesterol content was detected in a few plasmas tested but, owing to the difficulty of obtaining large volumes of blood from the rats, complete analyses of the other lipid fractions could not be performed. " Clearing " occurred within an hour of injection.

Investigations in man

Three male patients aged 42, 47 and 56 with coronary thrombosis were treated with heparin in the usual way immediately after infarction. In each man, there was a change in plasma lipoprotein to the faster-moving α-type ; this was particularly marked in the patient R. M—, who had lipaemia with an excess of β-lipoprotein. The phenol-reacting lipid rose slightly, but there was no significant change in cholesterol or phospholipid (Table III). The transformation of lipoprotein was maintained for 24 hr. in each case.

Injection of dextran sulphate (5000 units) into a fourth male patient A. G—, aged 52, with hyperlipaemia and myocardial ischaemia, caused similar changes, beginning in three minutes and being very marked after 20 min., at which time

TABLE III.—*Lipid Fractions of Plasma in Patients Before and After Injections of Sulphated Polysaccharides*

Patient	Diagnosis	Injection	Optical density (Extinction at 680 mμ)	Lipid fractions		
				Phenol-reactive g.%)	Cholesterol (g.%)	Phospho-lipids (g.%)
L. G—, ♂, 56	Coronary th.	Heparin, 12,000 u.	—	0·59/0·65	0·22/0·22	0·32/0·33
R. M—, ♂, 42	,, ,,	Ditto	—	0·71/0·78	0·26/0·22	0·35/0·35
A. G—, ♂, 52	Myocardial ischaemia	Dex. sulph., 5,000 u.	0·04/0·40	0·76/0·84	0·37/0·40	0·44/0·44
J. H—, ♂, 47	Coronary th.	Heparin, 12,000 u.	—	0·38/0·38	0·21/0·25	0·24/0·21
I. M—, ♀, 60	Myxoedema	Dex. sulph., 5,000 u.	0·07/0·22	0·50/0·93	0·28/0·30	0·29/0·29
P. J. A—, ♂, 56	Nephrosis	Dex. sulph., 3,000 u.	0·02/0·24	0·71/0·86	0·42/0·46	—

the proportion of α-lipoprotein had risen from 50 per cent to 81 per cent of the total (Fig. 6), producing an electrophoretic pattern like that seen in a woman or much younger man. At this point, the plasma on standing became turbid with an eight-fold rise in optical density, and a striking deposition of particulate fat, which readily clumped and coalesced. Two hr. after the injection of dextran sulphate, this patient was given a fatty meal (an omelette made with two eggs spread upon buttered toast). Four hr. later, when the plasma lipid total was almost double, the predominance of α-lipoprotein induced by the dextran sulphate still persisted (Fig. 6). The cholesterol level, already high, was not significantly altered. Similar changes were observed in a fifth (female) patient aged 60 with myxoedema in whom the lipaemia caused by a fatty meal was "cleared" and the α-lipoprotein moiety conferred by heparin injected 4 hr. previously, was retained.

In a sixth (male) patient with a nephrotic syndrome, a smaller dose (3000 units) of dextran sulphate caused immediate turbidity in plasmas sampled at 3 and 20 min. after injection, and kept at 37°. A fibrinoid clot then separated in the 20 min. sample, into which most of the particulate fat became incorporated, leaving a clear serum. Electrophoresis, performed immediately on separation of the plasmas, showed that the β-lipoprotein had again acquired α-type mobility with, in this instance, a fairly conspicuous pre-albumin component and some "trailing" of the albumin (Fig. 7).

Nature of the physico-chemical changes

In all the investigations here reported, whether in rabbits, rats or man, the most lasting effect observed after injection of heparin or dextran sulphate was the transformation of a variable proportion of the plasma lipoprotein into a fraction with greater electrophoretic mobility. In man and in the rabbit, this meant mainly a transformation from β- to α-type lipoprotein but in the rat the transference was from a pre-existing α-complex to a fraction which moved faster than the serum albumin, a change which, though present, was much less conspicuous in man. The plasma cholesterol, in each species, behaved similarly: there was no consistent or significant alteration in total cholesterol, but the bulk of that

present became more mobile electrophoretically and showed, by zone staining, a peak just behind the transformed lipoprotein front (Fig. 5 and 6).

The second component of the change in man and in the rabbit was the gradual separation of particulate fat some of which coalesced to produce chylomicron-like droplets of neutral fat and gradually caused the serum or plasma to become turbid on standing. The extent of this change depended largely upon the temperature and time after separation—as well as that after injection—at which the plasma was examined. Maximum turbidity developed in samples withdrawn 3–20 min. after injection showing, on immediate separation, a slight increase in optical density which intensified at 37° or, more markedly, at room temperature or 4°, within the next 4–24 hr., and then began to clear. Samples withdrawn 3 hr. after injection, or thereafter, usually showed some clearing and reduction in optical density. The third manifestation of physico-chemical change was an increase, beginning immediately after injection and lasting some hours, of phenol-reacting lipids. According to Kunkel *et al.* (1948), the turbidity developed in this reaction more or less defines the total lipid content of the serum but, in the results obtained here, the various lipid fractions were not significantly raised. All these changes were also observed, though in lesser degree, in non-lipaemic patients and animals.

Experiments in vitro

Heparin, dextran sulphate and dextran failed to produce any significant alterations in optical density and lipoprotein pattern when added *in vitro* to human or rabbit sera, whether normal or lipaemic. Addition of crystalline albumin or citrate to the post-heparin samples of plasma did not lessen the turbidity.

A number of other substances, mainly detergents, were also tested *in vitro* by a technique previously described (Lloyd and Stewart, 1956). Increase in lipoprotein mobility could be obtained somewhat uncertainly with anionic detergents but appeared to be part of a general increase in the electrophoretic mobility of all charged protein and lipid fractions in the serum. Conversely, retardation of mobility was obtained, firmly and reproducibly, with cationic detergents of various types (Fig. 8) some of which (*e.g.*, di-octyl cetyl pyridinium bromide) produced also immediate denaturation of all the lipoproteins. Non-ionic detergents were either inert, or caused interconversion of α- to β-type lipoprotein in the case of compounds with a certain length of ethenoxy chain.

Despite these striking changes in sera *in vitro*, injection of doses up to the toxic limit in rabbits of these substances produced only slight changes in lipoprotein pattern hardly comparable with those seen *in vitro*.

DISCUSSION

The well-known clearing action of heparin, *in vivo* as *in vitro* is almost certainly attributable to hydrolysis of triglycerides by an intermediate enzyme system (French, Robinson and Florey, 1953 ; Korn, 1957). In the experiments reported here, in the rabbit and man, the immediate effect of both heparin and dextran sulphate was the reverse of clearing, in that the plasma sampled within the first half-hour after injection became turbid at room temperature due to a rapid separation of particulate fat. At the same time, there was an apparent increase in total phenol-reacting lipids and some interconversion of slow-moving β- to

faster-moving α-lipoproteins on electrophoretic zone analysis. This interconversion persisted, though " clearing " began to occur within the hour.

The absence of an immediate clearing effect is not inconsistent with existing data, as Shore, Nichols and Freeman (1953) have shown that clearing depends upon the presence of triglycerides in quantity. *In vitro*, particularly when rat chyle is used, and in alimentary lipaemia, the substrate is therefore amenable to clearing. In the cholesterol fed rabbit and in man, the bulk of the lipaemia under the conditions described here was attributable to cholesterol and phospholipids in a stable β-lipoprotein complex of small molecules which probably contributed little to the turbidity of the plasma (Edsall, 1947 ; Lever and Hurley, 1953). The effect of heparin upon this complex was to cause immediate separation of chylomicron-like particles which were then in the classical fashion " cleared ".

Changes in the density and electrophoretic mobility of lipoproteins after sulphated polysaccharides have been reported by various workers (*e.g.*, Gofman *et al.*, 1954 ; Dangerfield and Smith, 1955 ; Jeavons and Ricketts, 1956). In a comprehensive review of the problem, Robinson and French (1957) consider that such changes are explained by physical effects of unesterified fatty acids from hydrolysed triglycerides, *i.e.*, a secondary effect of clearing. The changes in lipoprotein occurred however before visible clearing and were accompanied by a significant change in cholesterol distribution (Fig. 6) though not in total content. The appearance of particulate fat was also inconsistent with the idea that the changes were entirely secondary to lipolysis. Such particles had the appearance of neutral fat droplets microscopically ; the presence of precipitated soaps and fatty acids was excluded by their insolubility in citrate or albumin.

A clue to these discrepancies is perhaps afforded by the inherent difference between the sera of rats on the one hand and rabbit and humans on the other. In the rat—and studies of the heparin clearing reaction have been made largely in the rat—the lipoprotein is largely of α-type. Injection of heparin causes some of this to move electrophoretically with the albumin. The association of albumin and fatty acids has a solubilising effect (Robinson and French, 1953) and immediate lipolysis ensues. Except in states of alimentary lipaemia, with triglycerides in excess, there is much less linkage between albumin and lipid in human or rabbit sera. Indeed, French, Robinson and Harris (1955) have shown elsewhere that the plasma of cholesterol-fed rabbits will inhibit the clearing of chyle *in vitro* by heparinised rat plasma.

The observed rise in phenol-reactive lipids could be due, if the usual empirical significance of the reaction applies in these circumstances, to a temporary increase in total lipid content (Kunkel *et al.*, 1948). This would be at variance with the other data, since the individual fractions and gravimetric acetone-alcohol extracts did not show comparable quantitative variation. It is probable that the reaction is influenced also by qualitative factors and, since the rise in readings occurred only when particulate fat was separating, it may be attributable mainly to a physical alteration of the lipid colloid.

In contrast to these short-lived changes, the interconversion observed between β- and α-lipoprotein was of long duration, especially when dextran sulphate was injected. It may therefore be independent of the anticoagulant effect and of the presence of fibrinogen which lasted for about 3 hours. Injection of protamine sulphate, which rapidly neutralised anticoagulant action, did not reverse at the same time all the changes in the lipid fractions.

None of these changes occurred when the polysaccharide dextran was injected. This suggests that the ionic charge, as well as the molecular configuration of heparin and dextran sulphate may be of importance; but, on the other hand, the injection of large doses of anionic and cationic substances with high surface-activity toward lipids were almost without effect, despite the marked activity shown by comparable dilutions of these substances *in vitro*. The effects obtained with heparin and dextran sulphate are unlikely therefore to depend primarily upon ionic charge but rather upon some additional mechanism such as conjugation with the enzyme-substrate complex involved (Korn and Quigley, 1957) in the action of lipoprotein lipase.

As in all studies of this kind, the changes are of necessity observed in blood removed from the unknown complicating or balancing factors in the milieu interieur. The fact that some of the changes are gradual, and more marked at lower temperature, suggests that an additional element of artefact may also be present. Nevertheless, such changes appear to depend firmly upon the presence of sulphated polysaccharide *in vivo*, and there is much to suggest that, in part at least, they represent real alterations or interconversions of circulating lipoprotein components.

SUMMARY

In the absence of alimentary lipaemia, intravenous injection of sulphated polysaccharides caused in patients and in experimental rabbits a gradual separation of particulate fat in plasma sampled a few minutes after injection.

An increase in electrophoretic mobility of lipoproteins, including the main cholesterol fraction, also occurred and persisted for some hours. Some interconversion of β- to α-type lipoproteins occurred in human and rabbit plasmas. In hypercholesterolaemic rats, the lipoprotein was transformed to a pre-albumin component. This change, though present, was less marked in man.

These changes were more marked in conditions of non-alimentary lipaemia and were absent *in vitro*. In animals the overall effects were not obtained, *in vivo* or *in vitro*, with various charged and surface-active substances.

This action of sulphated polysaccharides, under the conditions described, occurs independently of classical " clearing " of triglyceride lipaemia and appears to cause an alteration lasting for some hours in the physical dispersion of lipid in the plasma.

Acknowledgment is made gratefully to the following : to the Medical Research Council for a personal and expenses grant ; to Dr. G. P. Baker for his interest and for access to patients under his care ; to Dr. J. M. Barnes for his interest and for facilities granted at the M.R.C. Laboratories, Carshalton ; and to Messrs. W. Archibald, P. Brooks, C. R. Kennedy and R. F. Legg for technical assistance.

The heparin used in these investigations was " Pabyrn ", supplied by Paines & Byrne, Greenford. The dextran sulphate used was " Dexulate ", supplied by Glaxo Laboratories, Ltd., Greenford, with an estimated polysaccharide content of between 50 and 60 mg. per ml.

REFERENCES

Bruce, H. M. and Parkes, A. S.—(1949) *J. Hyg., Camb., XLVII*, 202.

Dangerfield, W. G. and Smith, Elspeth B.—(1955) *Biochem. J.*, **59**, 6.

Edsall, J. T.—(1947) *Advanc. Protein Chem.*, **3**, 460.

Fillios, L. C., Andrus, S. B., Mann, G. V. and Stare, F. J.—(1956) *J. exp. Med.*, **104**, 539.

French, J. E., Robinson, D. S. and Florey, H. W.—(1953) *Quart. J. exp. Physiol.*, **38**, 101.

Idem, Robinson, D. S. and Harris, P. M.—(1955) *Ibid.*, **40**, 320.

Gofman, J. W., Delalla, O., Glazier, F., Freeman, N. K., Lindgren, F. T., Nichols, A. V., Strisower, B. and Tamplin, A. R.—(1954) *Plasma*, **2**, 413.

Hahn, P. F.—(1943) *Science*, **98**, 19.

Jeavons, S. M. and Rickets, C. R.—(1956) *J. clin. Path.*, **9**, 255.

King, E. J.—(1951) 'Micro-analysis in Medical Biochemistry'. London.

Korn, E. D.—(1957) *J. biol. Chem.*, **226**, 827.

Idem and Quigley, T. W.—(1957) *Ibid.*, **226**, 833.

Kunkel, H. G., Ahrens, E. H. and Eisenmenger, W. J.—(1948) *Gastroenterology*, **11**, 499.

Lever, W. F. and Hurley, Nancy A.—(1953) In ' Blood Cells and Plasma Proteins'. Ed. Tullis, New York.

Lloyd, G. A. and Stewart, G. T.—(1956) *J. clin. Path.*, **9**, 251.

Popjak, G.—(1946) *Biochem. J.*, **40**, 608.

Robinson, D. S. and French, J. E.—(1953) *Quart. J. exp. Physiol.*, **38**, 233.

Idem.—(1957) *Ibid.*, **42**, 15.

Shore, B., Nichols, A. V. and Freeman, N. K.—(1953) *Proc. Soc. exp. Biol., N.Y.*, **83**, 216.

Zilversmitt, D. B. and Davis, A. K.—(1950) *J. Lab. clin. Med.*, **35**, 155.

EXPLANATION OF PLATE

Fig. 1.—Effect of heparin (1,000 units/kg. intravenously) upon the electrophoretic pattern of the serum lipids in a hyperlipaemic rabbit.

Fig. 6.—Effect of intravenous dextran sulphate (5000 units) upon the electrophoretic pattern of the serum lipoproteins in a patient with myocardial ischaemia and hyperlipaemia.

Fig. 7.—Effect of intravenous dextran sulphate (3000 units) upon the electrophoretic pattern of the plasma lipoproteins in a patient with the nephrotic syndrome. (The protein bands, shown for position, remained unvaried.)

Fig. 8.—Effect of detergents upon the electrophoretic pattern of the serum lipoproteins *in vitro*. Each substance was tested at a final concentration of 0·1 per cent.

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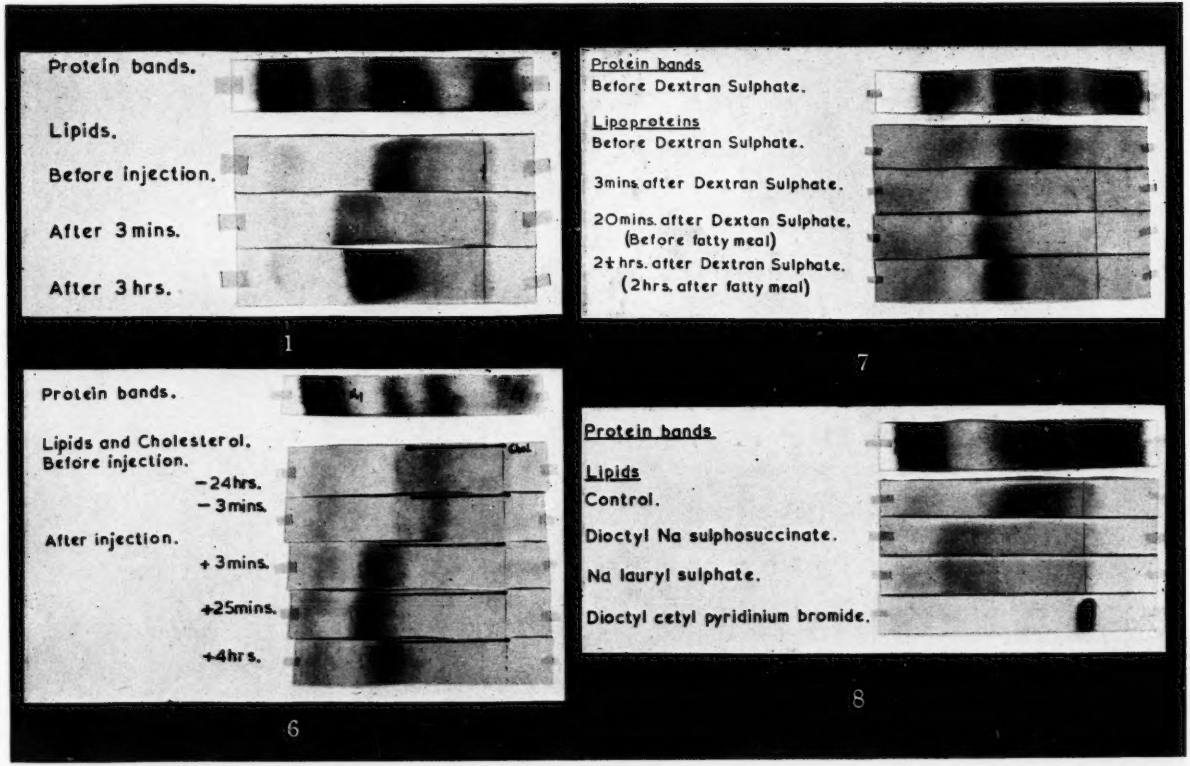
Protein bands.
Lipids.
Before injection.
After 3 mins.
After 3 hrs.
1
Protein bands
Before Dextran Sulphate.
Lipoproteins
Before Dextran Sulphate.
3mins. after Dextran Sulphate.
20mins. after Dextan Sulphate.
(Before fatty meal)
2½ hrs. after Dextran Sulphate.
(2hrs. after fatty meal)
7
Protein bands.
Lipids and Cholesterol.
Before injection.
−24hrs.
−3mins.
After injection.
+3mins.
+25mins.
+4hrs.
Chol.
6
Protein bands
Lipids
Control.
Dioctyl Na sulphosuccinate.
Na lauryl sulphate.
Dioctyl cetyl pyridinium bromide.
8

Stewart.

THE EFFECTS OF AMINO-ACETONITRILE AND CORTISONE ON THE HEALING OF TURPENTINE-INDUCED ABSCESSES IN THE RAT

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SINCE Ponsetti and Baird (1952) demonstrated that diets containing large amounts of the garden sweet pea (*Lathyrus odoratus*), in addition to their previously known effects on bone (Geiger, Steenboch and Parsons, 1933), produced gross damage to the aortic wall of the rat, many papers have appeared on various aspects of this subject. This work has been reviewed by Selye (1957). The toxic factor has been isolated and identified as N-γ-L-glutamyl-β-amino-propionitrile, and it has been shown that a series of related compounds, chiefly nitriles, have a similar action. However, the mechanism of their action which appears to be a widespread one on developing connective tissues is still obscure. In an attempt to gain further information of this action, it was decided to study the developing wall of an experimentally induced abscess in the rat and the changes produced in it by administration of the most potent of known lathyrogens, amino-acetonitrile (A.A.N.) and, further, to compare any changes observed with the known effects of cortisone on such abscess walls (Taubenhaus and Amromin, 1950).

MATERIALS AND METHODS

Sprague-Dawley and Wistar rats of either sex, both young (mean weight 50 g.) and adult (mean weight 150–200 g.) were used.

The animals were fed a diet of Baristoc—a local proprietary food pellet—supplemented by fresh green vegetables. This diet and water were supplied *ad libitum*.

Abscesses were induced by the injection of 0·25 ml. of turpentine and 0·75 ml. of air subcutaneously into the scapular region posteriorly.

A.A.N. was synthetized as its hydrosulphate by the method of Anslow and King (1941).

Both A.A.N. and cortisone acetate were injected daily subcutaneously into the inner side of the thigh: A.A.N. dissolved in distilled water at a concentration of 100 mg. per 1 ml., and cortisone acetate as a suspension in a concentration of 50 mg. per 1 ml. Injections were begun on the same day as the turpentine was administered.

At intervals varying from 2 to 19 days after the injection of turpentine the animals were killed by a blow on the head or by ether anaesthesia and the area of the abscess and overlying skin dissected from the deeper tissues and fixed immediately in 10 per cent formol saline. After fixation the abscess was dissected from the skin and strips cut from the deep aspect of its wall in a plane vertical to the skin surface. These were embedded in paraffin in the usual way and sections cut at 5–7 μ in thickness.

The following stains were used:

1. Haematoxylin and eosin—the eosin used is made in this Department and the details have been recently published by Menzies and Mills (1957). It differentiates Alcian blue very efficiently.
2. Van Gieson.
3. Gomori's modification of Masson's trichrome.
4. Alcian blue (Steedman, 1950).
5. Periodic acid Schiff (P.A.S.) (McManus, 1948), without the acid-reducing rinse of Hotchkiss (1948).

6. Ammoniacal silver for reticulin (Gomori as modified by Lillie, 1948).
7. Hale's colloidal iron as modified by Rinehart and Abul-Haj (1951).

Contraction temperatures were determined by cutting a strip approximately 3 × 0·2 cm. from the wall of the unfixed abscess, suspending it with a small weight attached in a beaker of water and applying heat gradually. The end point was quite sharp, the strip contracting to half to two-thirds of its initial length over a temperature range of 1–2°.

Capillary permeability was estimated by intravenous injection of 0·5 ml. of a 1 per cent solution of Evan's blue into a rat, followed immediately by the intradermal injection of 0·1 ml. of the substance to be tested into the previously shaved abdominal skin. The changes in the area of intradermal injection were followed for up to one and a half hours after injection.

RESULTS

Four groups of experiments were undertaken :

I. Control animals ;

II. animals receiving A.A.N.—10 mg. per day to young rats and 20 mg. per day to adult animals ;

III. animals receiving cortisone acetate—5 mg. per day to both young and adult rats ;

IV. animals receiving both A.A.N. and cortisone simultaneously in the same dosage as groups II and III.

In all groups an attempt was made to study the abscess over as long a period as possible but it was found that only occasionally could observations be extended beyond fourteen days as the abscesses discharged through the overlying skin at or before this time and study of the residual collapsed wall was not satisfactory. An impression was gained that such discharge occurred earlier and more frequently in animals receiving A.A.N., but the results were too variable to allow of certainty on this point.

In all the young animals treated with either A.A.N. or cortisone, and especially with the two drugs simultaneously, gross retardation of growth occurred. In addition, the typical skeletal deformities of lathyrism developed rapidly in young animals of groups II and IV, a number being grossly deformed within fourteen days. No case of aortic rupture occurred.

Adult animals did not show either of these changes but a loss of coat lustre and generally apathetic behaviour were seen in animals receiving A.A.N. for seven days or more.

A small number of young animals from each of group II, III and IV died during the period of experiment—some from demonstrable secondary infection, others without obvious cause.

The number of animals surviving the experiment and available for histological examination is shown in Table I.

TABLE.—*Number of Animals Used Subdivided According to Age and Drugs Administered*

Group	Drug administered	Number of animals		
		Young	Adult	Total
I	None (control)	3	9	12
II	A.A.N.	8	9	17
III	Cortisone	6	5	11
IV	A.A.N. + Cortisone	6	4	10

No difference in the development of the abscess was seen between young and adult animals in either control or injected groups, and the findings will be treated together. It is convenient to describe the changes seen in the four groups in order.

Abscesses in control animals

Macroscopic.—After two days the abscess was well defined from the surrounding tissue, having a thin transparent wall through which yellow pus could be clearly seen. By the fourth day a firm white opaque wall was present, and this progressively thickened until on removal all the later abscesses appeared as firm, roughly spherical, white masses adherent to the deeper surface of the skin (Fig. 1A).

Microscopic.—At two days the wall consisted of necrotic material centrally, surrounded by a thick layer of polymorphonuclear leucocytes, showing degeneration and necrosis adjacent to the central necrotic area. Occasional macrophages were seen in the outer part of this leucocytic layer, which externally abutted directly on skeletal muscle.

By the fourth day the wall had increased considerably in total thickness, and from within out the following layers were seen :

i. Necrotic material ;

ii. a zone of polymorphonuclear leucocytes, considerably thinner than that seen on day 2 ;

iii. a layer of granulation tissue composed of macrophages, capillary loops and fibroblasts, with some polymorphs especially in its inner portion. The axis of the capillary loops and fibroblasts was perpendicular to the surface of the abscess.

Some fine fibres, staining black by silver impregnation, pink with P.A.S. and pink with van Gieson, were seen in the outer half of this layer, embedded in material staining pink with eosin or P.A.S. ;

iv. a layer of active fibroblasts with clearly visible fibres between them, both cells and fibres regularly orientated parallel to the abscess cavity—that is, at right angles to the preceding layer.

Fibres in this layer stained pale pink with P.A.S., black by silver impregnation and deep red with van Gieson, and large P.A.S.-positive granules were present in the fibroblasts and macrophages.

The relative thickness of layers ii, iii and iv was as 1 : 2 : 1.

By the seventh day the same four layers were still present, but their proportions had altered considerably, now appearing as 1 : 1 : 2 from within out (Fig. 1B).

The fibrillar content of the two outer layers had increased considerably, but their staining characteristics had not altered except that the fibres in the outermost part of the wall had thickened and stained brown rather than black with silver impregnation.

On the ninth day only a few polymorphonuclear leucocytes were present in the inner part of the wall, the granulation tissue layer was relatively thinner and the greater part of the wall was composed of concentrically arranged fibroblasts and fibres, thin in the inner portion and thicker externally (Fig. 1C). These fibres all stained pink with P.A.S. but the reaction to silver changed from black to brown and the intensity of staining with van Gieson increased from within out.

From this time until observation ceased on the fourteenth day these changes steadily progressed—the outer concentric layer occupying an increasing proportion of the whole wall and its fibres becoming thicker and assuming the staining properties of collagen rather than of reticulum (Fig. 5). The intervening cells concurrently became less active in appearance—the nuclei decreasing in size and staining more deeply and the cytoplasm, with its P.A.S.-positive granules, becoming less apparent (Fig. 6). Some mature fibroblasts were present in the outer part of the wall as early as the seventh day (Fig. 1C).

At all stages after the fourth day the intercellular substance in the two outer layers, relatively small in amount, stained positively to both Alcian blue and Hale's colloidal iron and a pale pink with P.A.S.

Abscesses in animals receiving A.A.N.

Macroscopic.—During the first four days the abscesses showed no significant differences from the controls. After this period the wall became opaque and white in the usual way, but the thickening of the wall seemed slower to develop than in the controls so that at all stages the wall appeared relatively looser and less opaque and had a softer consistence to touch.

In addition, from the fourth day onwards a prominent red margin appeared in the outer part of the wall (Fig. 2A) and this persisted until at least the fourteenth day. No corresponding structure was seen in the controls.

Microscopic.—For the first two days the reaction appeared identical with that seen in the control animals, except that in some areas polymorphonuclear leucocytes appeared to penetrate more deeply into the surrounding tissues.

Definite differences first became apparent about the fourth day, and were seen in the outer part of the wall—layer (iv) as described in the control.

At first the changes were slight but with time they became steadily more apparent. At all stages the changes were similar in type.

The layer of forming fibrous tissue was of normal or slightly increased thickness and fibroblasts were present in not less than their normal numbers (Fig. 2B). They were normal in appearance and contained obvious P.A.S.-positive granules, but their maturation was delayed so that at all stages the proportion of mature cells was relatively decreased (Fig. 2C) and as late as the tenth day the majority still possessed active nuclei and abundant cytoplasm containing P.A.S.-positive granules. Their regular arrangement, concentric with the cavity of the abscess, was lost and they appeared to follow no regular pattern (Fig. 7 and 8).

Between the cells was an apparent increase of intercellular substance of considerable degree—this material stained pale pink with P.A.S., positive to Alcian blue and to colloidal iron. The fibres embedded in it had also lost their concentric arrangement and whilst silver-positive reticular fibres were seen in normal numbers in the inner part of the zone, their usual aggregation into dense concentric bundles in the outer layers was not apparent in many areas, fine fibres of irregular arrangement persisting to the periphery of the abscess. These stained positively with van Gieson but still gave a black reaction after silver impregnation.

Capillaries were more prominent than usual in the full thickness of the fibroblast layer, accounting for the red margin seen macroscopically (Fig. 2B). No abnormality in the macrophages was detected.

Abscesses in animals receiving cortisone

Macroscopic.—By contrast with the preceding two groups localization of the abscess was very poor. Instead of small white spherical masses, large flat areas were seen (Fig. 3A), surrounded as late as the eleventh day by a thin transparent wall through which yellow pus was clearly visible.

Microscopic.—At four days the wall consisted of a layer of polymorphonuclear leucocytes of similar thickness to that of controls of the same period, with only scattered macrophages between it and underlying muscle.

By the seventh day the wall was approximately one half the thickness of the control and consisted of polymorphs and macrophages surrounded by a thin layer of fibroblasts and relatively mature fibrous tissue (Fig. 3B and C). A few dilated capillaries were seen in this layer.

Over the next few days the fibrous tissue layer gradually thickened, but by the eleventh day when observation ceased, it was not more than a small fraction of the thickness of the wall of the control.

At no time did a definite layer of granulation tissue develop internal to the fibrous layer, which appeared to be derived in part from compression of pre-existing collagen fibres in the area.

A few dilated capillaries remained in this layer on the eleventh day.

Abscesses in animals receiving A.A.N. and cortisone

Macroscopic.—These closely resembled those in group III, differing only in spreading even more widely and having still thinner walls and, more obviously, in the presence of a red line of dilated capillaries in the outer part of their wall (Fig. 4A). This was clearly seen in all abscesses of the group.

Microscopic.—The changes seen closely resembled those in animals receiving cortisone alone. The fibrous layer external to the inflammatory cell zone was thinner than in the preceding group (Fig. 4B) and in some areas consisted of fine irregular fibrils staining black with silver impregnation and positively with van Gieson in place of the thicker, more regular bundles of group III, which showed the typical staining reactions of collagen.

Slight increase of the intercellular substance, staining as in the animals receiving A.A.N. alone, was seen in these areas.

Dilated blood vessels were a prominent feature of the outer part of the wall in every case (Fig. 4C).

Contraction temperatures of abscess walls

Strips were tested from the wall of abscesses in all four groups on the fourth, seventh, ninth and eleventh day after turpentine injection. In all cases contraction occurred in the range 65–68°, with no detectable difference between the four groups.

Local Effect on Capillary Permeability

A solution of A.A.N. sulphate in the concentration used for subcutaneous injection, and 10- and 100-fold dilutions of this solution, were tested concurrently with a control solution of normal saline.

In the two higher concentrations a spreading weal developed rapidly and reached a diameter of 2 cm. within thirty minutes ; a well-defined band of blue

was present around the weal. The 1/100 dilution showed a similar but smaller response and the saline control produced neither weal nor blue band.

Estimation of the pH of the A.A.N./sulphate solution gave a value of 1·5–2·0; the experiment was repeated with the pH of the A.A.N. solution adjusted to 6·5–7·0 by addition of a veronal buffer. After this alteration of the pH neither weal nor blue band was apparent with any of the A.A.N. solutions.

DISCUSSION

From the experiments described there is no doubt that A.A.N. has a demonstrable effect on the developing abscess wall.

This seems not to be one affecting cellular proliferation, and in particular not one involving the multiplication of fibroblasts. The effect seems rather to lie in the maturation of these cells and in the formation of collagen and is accompanied by a loss of the normal regular orientation of cells and fibres in the abscess wall.

This is in agreement with several known observations regarding the action of A.A.N. and related compounds on connective tissue. Enzinger and Warner (1957) found considerable reduction in the tensile strength of healing incised wounds in the abdominal wall of the rat, most clearly demonstrable on the fourth and fifth day of healing. In a histological study of these wounds they found no delay in fibroblastic proliferation, but weakness and irregularity in collagen fibre formation accompanied by unduly long persistence of P.A.S.-positive intercellular substance normally present in the earlier stages of wound healing.

Mielke, Lalich and Angevine (1957), in a study of the effect of β-amino-propionitrile on croton oil induced subcutaneous pouches in the rat, reported poor formation and collapse of the pouches and were able to demonstrate decreased production of hydroxyproline in the pouch wall, hexosamine production being apparently normal. In an accompanying histological study, not completely satisfactory apparently due to the necessary chemical treatment of the wall before fixation, they reported increase in fibroblast numbers, decrease in collagen fibres, a general loss of orientation in the wall and an apparent increase in intercellular substance. They concluded that β-amino-propionitrile in some way interferes with collagen synthesis.

Evidence that fibroblast proliferation is not impaired, but is rather stimulated was provided by Yeager and Hamre (1957), who found definite proliferation of fibroblasts in exostoses of the adult rat femur within twenty-four hr. of commencing a diet containing 50 per cent *Lathyrus odoratus* meal.

Indirect evidence that connective tissue formation is the site of action rather than any change in formed connective tissue is provided by the observation that gross skeletal deformities, aortic rupture and other features of the experimental lathyrous syndrome only occur in growing animals, adults being relatively insusceptible to even high dosage of A.A.N. and allied compounds. By contrast the growing tissue in a healing wound or abscess wall appears equally susceptible in young and mature animals. Whilst all the above work leads to the same general conclusion regarding the essential nature of the lathyrous lesion, the study of the development of the bony and aortic lesions has not led to similar clarity. Menzies and Mills (1957) concluded that the basic change was " an accumulation of chondroitin sulphate together with a general breakdown of supporting structures such

as collagen fibres ". By contrast, Churchill, Gelfant, Lalich and Angevine (1955), while finding a similar chondroitin sulphate increase also described a primary degeneration of elastic fibres, in which finding they are supported by Walker and Wirtschafter (1956) whose illustrations however are not convincing, as in some, pre-existing smooth muscle cells appear to have been confused with " fibroblastic proliferation ".

The technical difficulties associated with study of the rat aorta, allied to the circumstance that, with the exception of the work of Yeager and Hamre (1957) quoted above, all the work on bone has been done on relatively chronic lesions, make it probable that the conclusions drawn from studies on wounds and abscesses indicate more accurately the basic change, but more work on both is needed to resolve the apparent anomalies.

The changes seen in granulation tissue are somewhat similar to those described in scurvy by Penny and Balfour (1949) and Bunting and White (1950). However, vitamin C is most unlikely to be involved in the mode of action of A.A.N. as Enzinger and Warner (1957) found administration of vitamin C made no difference to the delay in wound healing caused by A.A.N., and heavy dosage of vitamin C fails to prevent the bony and aortic lesions of lathyrism (Lewis, Fajans, Esterer, Shen and Oliphant, 1948). This finding was confirmed in work carried out in this Department during 1955. Direct comparison of the two lesions in the rat is not possible as scurvy cannot be produced experimentally in this species.

In view of the doubt regarding the histochemical specificity of the various stains for mucopolysaccharides and related compounds (Meyer, 1953), and the incompleteness of our knowledge of the intimate structure of collagen fibres and their relation to fibroblasts (Jackson, 1956), a discussion of the nature of the change on a histochemical rather than a histological descriptive plane is of little value.

The evidence from our experiments on the local effect of A.A.N. on capillary permeability suggests that there is no significant local action on small blood vessels and the striking zone of capillary dilatation seen around abscesses in animals receiving A.A.N., either alone or with cortisone, may be a consequence of the delay in fibrous tissue maturation rather than a direct effect of the drug. The local haemorrhage at the site of injection reported by Selye (1957), and repeatedly confirmed by us, is almost certainly due to the high degree of acidity of the solution administered and not to the drug itself.

The changes observed in this series following the administration of cortisone are in general agreement with the known effects of this substance applied either locally or systemically to granulation tissue (Ragan, Howes, Plotz, Meyer and Blunt, 1949; Taubenhaus and Amromin, 1950). The changes seen are even more gross than those observed by these authors; but a higher dose of cortisone was used.

The type of change is quite distinct from that produced by A.A.N. and consists of a gross decrease in the rate of multiplication of fibroblasts with a consequent diminution in the amount of granulation tissue and collagen formed. We saw no evidence of the abnormal accumulation of macrophages described by Taubenhaus and Amromin, but the primary polymorphonuclear response appeared unimpaired.

In the animals who received both A.A.N. and cortisone the dominant effect observed was that due to cortisone. However, the changes described above as characteristic of A.A.N. administration were seen in what little fibrous wall

developed in the abscesses of this group, and the peripheral red zone of vascular dilatation was very obvious in these animals in striking contrast to the reduced vascularity of the abscesses in animals receiving cortisone alone.

It would appear that the two drugs produce effects on granulation tissue independently of one another.

The failure to detect any change in contraction temperature of the abscess wall in animals receiving either A.A.N. or cortisone indicates that what collagen does form is in each case normal, in this respect at least, as it can be assumed that heat contraction of the wall is due to its collagen content.

The factors influencing such contraction have been recently reviewed by Gustavson (1956).

SUMMARY

The effect of systemic administration of A.A.N. and cortisone, alone and together, on the healing of turpentine-induced abscesses in the rat is described.

The effect of A.A.N. is to cause a delay in the laying down of mature collagen in the wall, accompanied by abnormal persistence of amorphous intercellular substance. Proliferation of fibroblasts is not impaired, but their maturation is delayed.

A.A.N. appears to have no local dilator effect on blood capillaries, nor does it increase capillary permeability.

The known effect of cortisone in grossly delaying fibroblastic proliferation is confirmed.

The effects of A.A.N. and cortisone on developing connective tissue appear to be independent of each other.

EXPLANATION OF PLATES

In each figure (A) is a photograph, actual size, of the opened abscess. (B) is a low power photomicrograph of a section of the whole thickness of the abscess wall, stained with haematoxylin and eosin. × 40. (C) is a photomicrograph of a section of the outer part of the wall in Fig. 1 and 2, and its whole thickness in Fig. 3 and 4, stained with haematoxylin and eosin. × 180.

FIG. 1.—Abscess in control animal at 7 days.

FIG. 2.—Abscess in animal receiving A.A.N. at 7 days. Note its approximately normal size and thickness of wall in (A) and (B), with prominent congested margin in (A), and also separation and loss of orientation of cells in outer part of the wall in (C).

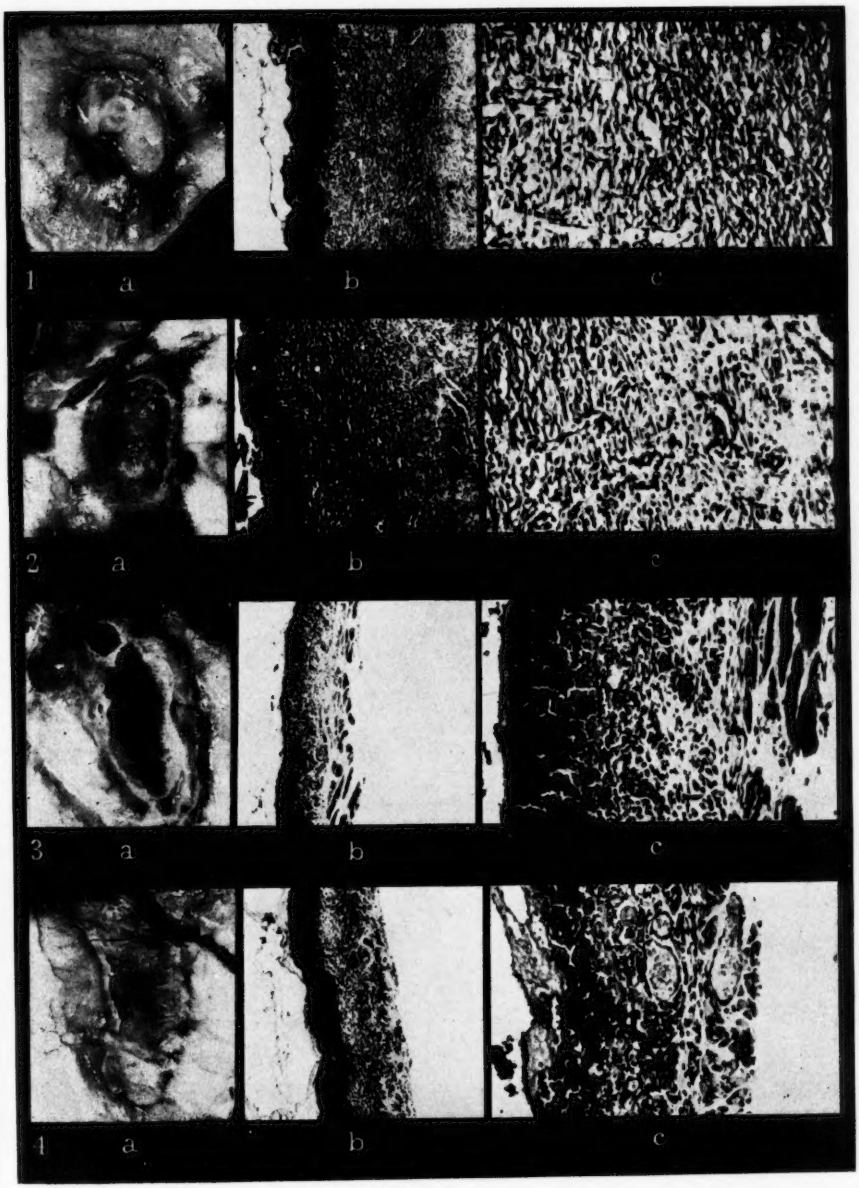
FIG. 3.—Abscess in animal receiving cortisone at 7 days. Note increase in size and the thinness of the wall in (A) and (B), and gross decrease of granulation tissue in outer part of wall in (C).

FIG. 4.—Abscess in animal receiving both A.A.N. and cortisone at 7 days. Note increase in size and extreme thinness of wall visible in (A), (B) and (C), almost complete absence of granulation tissue and presence of large dilated blood vessels in outer part of the wall in (C).

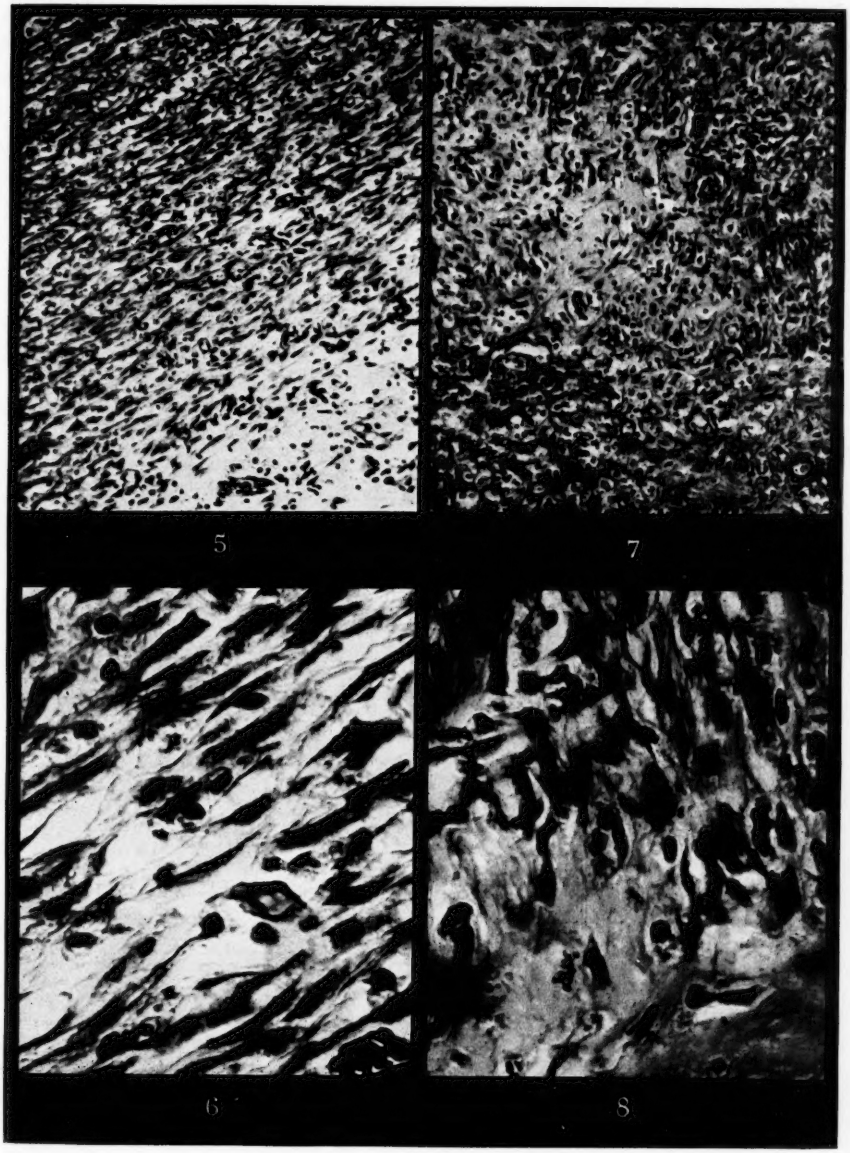
FIG. 5 and 6.—Photomicrographs of portion of a section of the wall of abscess in control animal at 12 days, stained with haematoxylin and eosin. Fig. 5 × 180 Fig. 6 × 960.

FIG. 7 and 8.—Photomicrographs of portion of a section of the wall of abscess in an animal receiving A.A.N. at 12 days, stained with haematoxylin and eosin. Fig. 7 × 180. Fig. 8 × 960.

Note absence of the normal concentric arrangement of fibroblasts and fibrils seen in Fig. 5 and 6, with relative immaturity of many of the cells and great increase of eosinophilic intercellular material.



Hurley, Storey and Ham.



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Failure to detect any alteration of the contraction temperature of the wall of any of the abscesses studied suggests that what collagen is formed in the presence of either A.A.N. or cortisone is normal.

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REFERENCES

ANSLOW, W. R. AND KING, H.—(1941) In 'Organic Synthesis', edited by Gilman, H. and Blatt, A. H., 2nd Ed. New York (John Wiley and Sons Inc.), coll. vol. 1, p. 258.
BUNTING, H. AND WHITE, R. F.—(1950) *Arch. Path.*, **69**, 590.
CHURCHILL, D. W., GELFANT, S. LALICH, J. J. AND ANGEVINE, D. M.—(1955) *Lab. invest.*, **4**, 1.
ENZINGER, F. M. AND WARNER, E. D.—(1957) *Ibid.*, **6**, 251.
GEIGER, B. J., STEENBOCH, H. AND PARSONS, H. T.—(1933) *J. Nutr.*, **6**, 427.
GUSTAVSON, K. H.—(1956) In 'The Chemistry and Reactivity of Collagen', New York (Academic Press Inc.).
HOTCHKISS, R. D.—(1948) *Arch. Biochem.*, **16**, 131.
JACKSON, S. F.—(1956) *Proc. roy. Soc.* B, **144**, 556.
LEWIS, H. B., FAJANS, RUTH S., ESTERER, MARIE B., SHEN, C. AND OLIPHANT, MARGARET.—(1948) *J. Nutr.*, **36**, 537.
LILLIE, R. D.—(1948) In 'Histopathologic Technique', Philadelphia (The Blakiston Co.), p. 179.
MCMANUS, J. F. A.—(1948) *Stain Tech.*, **23**, 99.
MENZIES, D. W. AND MILLS, K. W.—(1957) *J. Path. Bact.*, **73**, 223.
MEYER, K.—(1953) In 'The Mechanism of Inflammation', edited by Jasmin, G. and Robert, A., Montreal (Acta, Inc.), p. 170.
MIELKE, J. E., LALICH, J. J. AND ANGEVINE, D. M.—(1957) *Proc. Soc. exp. Biol., N.Y.*, **94**, 673.
PENNY, J. R. AND BALFOUR, BRIGID M.—(1949) *J. Path. Bact.*, **61**, 171.
PONSETTI, I. V. AND BAIRD, W. A.—(1952) *Amer. J. Path.*, **28**, 1059.
RAGAN, C., HOWES, E. L., PLOTZ, C. M., MEYER, K. AND BLUNT, J. W.—(1949) *Proc., Soc. exp. Biol., N.Y.*, **72**, 718.
RINEHART, J. F. AND ABUL-HAJ, S. K.—(1951) *Arch. Path.*, **72**, 189.
SELYE, H.—(1957) *Rev. canad. Biol.*, **16**, 1.
STEEDMAN, H. F.—(1950) *Quart. J. micr. Sci.*, **91**, 477.
TAUBENHAUS, M. AND AMROMIN, G. D.—(1950) *J. Lab. clin. Med.*, **36**, 7.
WALKER, D. G. AND WIRTSCHAFTER, Z. T.—(1956) *Arch. Path.*, **61**, 125.
YEAGER, V. L. AND HAMRE, C. J.—(1957) *Ibid.*, **64**, 171.

EFFECT OF NEW-BORN RABBIT AND MOUSE LIVER SUSPENSIONS ON X-IRRADIATED RABBITS*

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PIONEER work by Jacobson (1952) and Lorenz, Uphoff, Reid and Shelton (1951) established that homologous or heterologous cell suspensions of blood-forming tissues when administered to lethally X-irradiated rodents can induce rapid haemopoietic recovery and increase survival.

Early evidence tended to favour the hypothesis that these beneficial effects were due to " humoral " stimulation by some unidentified factor causing accelerated regeneration of the host marrow (Jacobson, 1952). However, the possibility that " cellular repopulation " of the depleted marrow by the injected donor cells might be the mechanism was repeatedly stated by Loutit (1954). Recently several reports have appeared giving direct evidence of such survival and proliferation of injected marrow and spleen cells in irradiated recipients (Ford, Hamerton, Barnes and Loutit, 1956 ; Nowell, Cole, Habermeyer and Roan, 1956 ; Lindsley, Odell and Tausche, 1955 ; Makinodan, 1956 ; Merwin and Congdon, 1956 ; Mitchison, 1956 ; Vos, Davids, Weyzen and Van Bekkum, 1956 ; Porter, 1957).

With the general acceptance of cellular repopulation as the main mechanism by which haemopoietic suspensions bring about their therapeutic effects, it was considered worthwhile to reinvestigate the reported beneficial effects on X-irradiated rabbits of liver cell suspensions from new-born rabbits (Jacobson, Marks and Gaston, 1956*a*) and mice (Jacobson, Marks and Gaston, 1956*b*).

In the experiments reported here it is shown that whereas rabbit liver causes some increase in the 56-day survival rate of X-irradiated rabbits, mouse liver does not. Further by using the sex difference in rabbit heterophils as a " label " (Porter, 1957) it is shown that the introduced blood-forming cells from new-born rabbit liver survive and repopulate the host's depleted haemopoietic tissue. Successful transplantation does not occur following treatment by mouse liver.

MATERIALS AND METHODS

Young adult male albino rabbits, which were not inbred in the genetic sense, weighing 2·8–3·2 kg. were used as recipient animals.

X-rays were produced in the first experiment by a 250 kvp. Phillips machine, in the second by a 250 kvp. Westinghouse machine, both operating at 15 mA, and with 0·5 mm. Cu and 1 mm. Al filters giving a half value layer in Cu of the filtered beam of 1·7 mm. The exposure rate was 93 r/min. at 50 cm. centre of animal to target, measurement being made in air with a Victoreen ionisation chamber, each animal receiving 900 r rotational total body irradiation.

The liver suspensions for post-irradiation injection were obtained as follows.

Homologous 1–3-day-old female rabbits.—After killing the donors by ether the liver was quickly removed and suspended gently in 10 ml. of cold saline. The number of nucleated cells per cu. mm. was determined by making a dilution with 2 per cent acetic acid in a white

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cell counting pipette and counting the cells on a standard haemocytometer. Such suspensions contained on the average 500 ($\times 10^6$) nucleated cells, one liver being used to treat each rabbit.

Heterologous 1–3-day-old CF No. 1 mice of both sexes.—The donors were killed by decapitation and the livers removed and suspended in 10 ml. of saline. Several livers were used to treat each rabbit, the cell suspension being adjusted so that each 10 ml. contained approximately 500 ($\times 10^6$) nucleated cells.

Injections of these cell suspensions were given within 30 min. of the death of the donor and were administered intravenously into the marginal ear vein of the recipient rabbit one to three hours following irradiation. Controls each received 10 ml. of saline intravenously.

Total leucocyte counts and microhaematocrits were determined on the irradiated animals every 2 days for the first 30 days and once per week thereafter. Blood films were taken from the ear at the same time intervals and in the rabbit liver experiment stained by Wright's method and the heterophils examined for nuclear " drumsticks ", as described previously (Porter, 1957). The number of such cells seen in examining 300 heterophils was recorded as percentage of the heterophil count. In the mouse liver experiment two films were taken from each rabbit, one being stained by Wright's method, the other by Gomori's method (1941) for alkaline phosphatase. Heterophils showing the characteristics of mouse neutrophils, i.e. faintly staining inconspicuous granules and a negative reaction for alkaline phosphatase, were then sought. (Rabbit " neutrophils " have conspicuous eosinophilic granules and are strongly positive for alkaline phosphatase.)

The rabbits were weighed daily for the first month, then at weekly intervals. All animals dying were autopsied, but the brain was not examined. Thymus, spleen, mesenteric lymph node and appendix were taken, fixed in formol-saline and routinely stained with haematoxylin and eosin. Marrow from the mid-point of the shaft of the right femur was taken from each animal, fixed in Helly's fluid and stained with haematoxylin and eosin. Marrow smears were prepared by the method of Berenbaum (1956) and treated with Wright's stain. In the mouse liver experiment additional samples of marrow were stained for alkaline phosphatase, the smears by Gomori's method, the sections by Nowell's technique (1957).

Experimental procedure

A total of 111 male New Zealand white rabbits were exposed to a single dose of 900 r whole body X-irradiation. Of these 36 (30 per cent) died in shock shortly after the irradiation. The surviving 80 animals were divided into 2 groups (Table I).

TABLE I.—*Effect of Neonatal Mouse and Rabbit Liver Treatment on survival of Rabbits Receiving 900 r Whole Body X-irradiation*

Group	Treatment	No. of animals	Alive at 14 days		Alive at 56 days	
			No.	Per cent	No.	Per cent
I	Saline	20	1	5	1	5
	Rabbit liver	20	10	50	5	25
II	Saline	20	2	10	1	5
	Mouse liver	20	3	15	2	10

Group I consisted of 40 animals, 20 of which received saline after irradiation whilst the other 20 were given female neonatal rabbit liver.

Group II consisted of a further 40 animals, 20 of which were controls and received saline after irradiation, the other 20 received baby mouse liver.

RESULTS

Group I

Those treated with neonatal rabbit liver.—The results for this group of 20 male rabbits are summarised in Table II.

A successful haemopoietic transplant was shown by 10 animals (50 per cent) as indicated by rapid restoration of the total leucocyte count to normal and the appearance in the peripheral blood of significant numbers of female heterophils

TABLE II.—*Analysis of Effects on 20 Male Rabbits of 900 r Whole Body X-irradiation followed by Intravenous Neonatal Rabbit Liver Suspension*

Outcome	No.		Per cent	
Failed to show a successful haemopoietic transplant—				
Died from gastric perforation	6	10	30	50
Died from infection	3		15	
Regenerated own bone marrow and survived	1		5	
Showed a successful haemopoietic transplant—				
Died from gastric perforation	3	10	15	50
Died at 19 and 25 days as result of early rejection of transplant by host	2		10	
Died from infection at 44th day with transplant intact	1		5	
Remained alive and well with haemopoietic transplant intact and functioning for 56 days	4		20	

bearing characteristic nuclear "drumsticks" (Fig. 1). Two of these animals subsequently died as the result of early rejection of the haemopoietic transplant by the host; one died at the 44th day following progressive wasting, diarrhoea

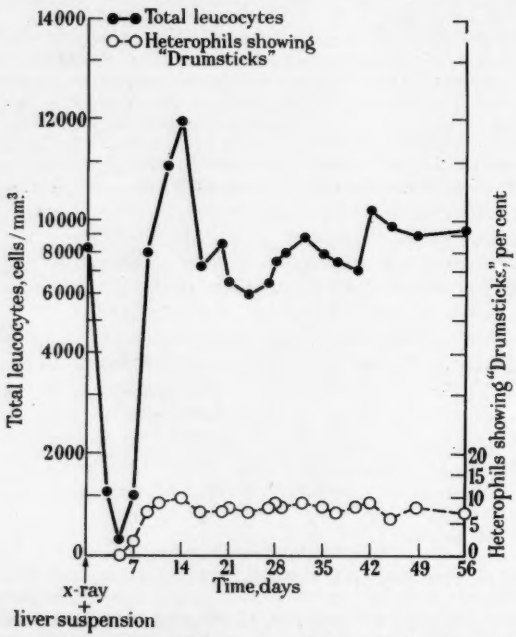
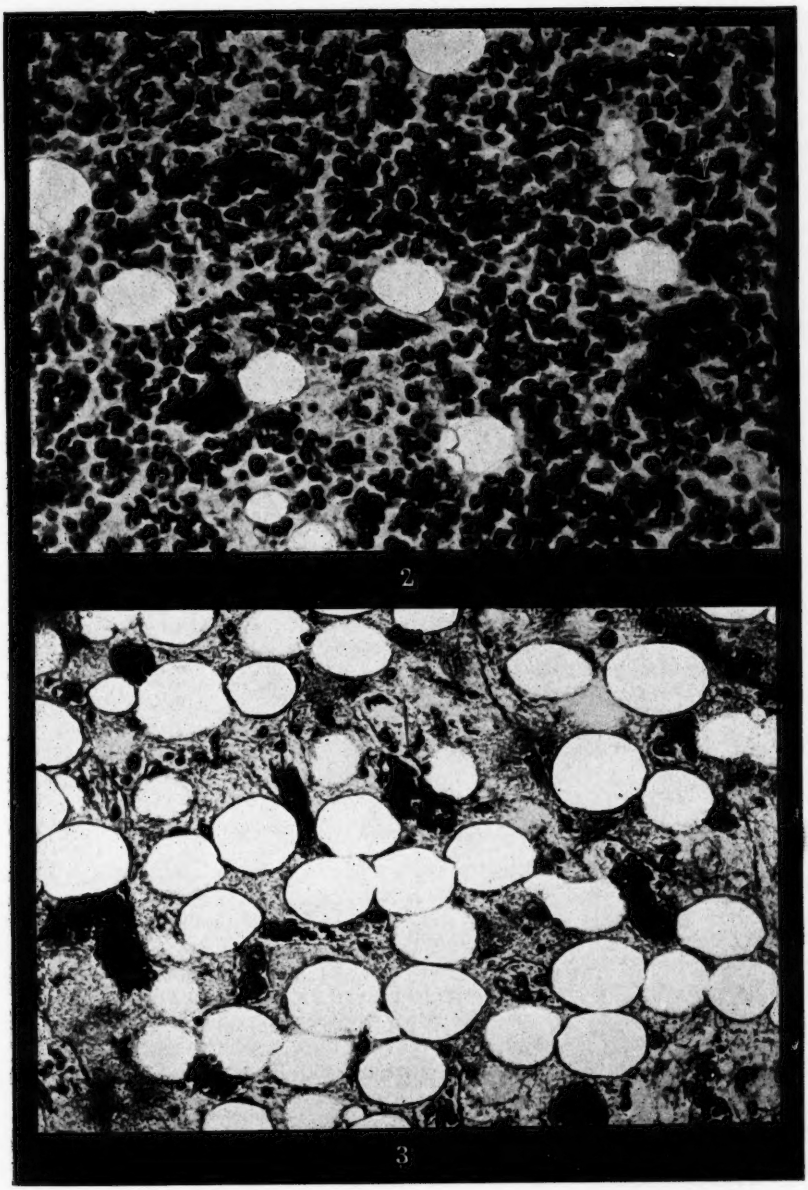


FIG. 1.—Appearance of female heterophils in peripheral blood of male irradiated rabbit after treatment with neonatal female rabbit liver suspension.

EXPLANATION OF PLATE

FIG. 2.—Femoral bone marrow from neonatal rabbit liver treated animal dying nine days after irradiation from perforation of a gastric ulcer. Haemopoietic transplantation had occurred as shown by the presence of female heterophils in the peripheral circulation and in marrow smears. Haematoxylin and eosin. × 400.

FIG. 3.—Aplastic femoral bone marrow from irradiated mouse liver treated animal dying ten days after irradiation from perforation of a gastric ulcer. The venous sinuses are distended with red cells and there is no haemopoietic regeneration. Haematoxylin and eosin. × 400.



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Porter and Mosely.

and leucocytosis, with marked lymphoid atrophy, but with an intact and functioning marrow transplant, probably due to an immune response of the graft against the host (Uphoff, 1957); three died from gastric ulcer perforation; and four survived the experimental period with the haemopoietic transplant intact and still contributing female cells to the peripheral blood.

Autopsy of animals from this group at the time of death or sacrifice showed the bone marrow to be normal or hyperplastic (Fig. 2) with female cells demonstrable in smears. The two cases which died as the result of early rejection of the haemopoietic transplant by the host were an exception: there was marked bone marrow destruction with only a few tiny islands of surviving haemopoietic cells.

Of the 10 animals which failed to secure a successful transplant only one regenerated its own bone marrow and survived.

Controls.—In the control group one animal survived the experimental period after regenerating its own bone marrow. Of the remainder, eight died from gastric perforation, four from intestinal haemorrhage and seven from infection, mainly bronchopneumonia: all showed marrow aplasia at autopsy (Fig. 3).

Group II

Those treated with neonatal mouse liver.—The results for this group did not differ materially from those obtained for the two control groups, only three animals surviving the experimental period after regenerating their own bone marrow. Of the remainder, six died from gastric perforation, three from intestinal haemorrhage and eight from infection. Marrow aplasia was present in all 17 at autopsy. In no instance were neutrophilic, alkaline phosphatase negative heterophils of mouse type seen in any of the peripheral blood smears obtained after irradiation, and it was concluded that heterotransplantation of mouse tissue had not occurred.

Controls.—In this group two animals survived after regenerating their own bone marrow, seven died from gastric perforation, four from intestinal haemorrhage, one from intussusception and six from infection.

Table I shows the numbers of animals surviving in each of these experimental groups at 14 and 56 days after treatment.

DISCUSSION

The results of these experiments confirm the ability, noted recently by Jacobson and his associates (1956*a*), of neonatal rabbit liver to improve the early survival rate of X-irradiated rabbits. This beneficial activity is now shown to be due to the presence of blood forming cells in the liver suspension, which are capable of surviving and giving rise to progeny which repopulate the host's depleted haemopoietic tissues and lead to rapid haematological recovery. These findings supplement those of Ford *et al.* (1956) which have led to the general recognition that the "protective" activity of spleen and bone marrow after radiation is directly related to their content of primitive haemopoietic elements. There now seems little reason to believe that a humoral factor is of importance following the use of any of these suspensions.

Failure of mouse neonatal liver preparations to similarly modify the response of X-irradiated rabbits was associated with inability of the mouse cells to persist and proliferate even temporarily in the new host, presumably because of the degree of genetic dissimilarity. This observation is at variance with an earlier report

that baby mouse liver enhances the survival of irradiated rabbits (Jacobson *et al.*, 1956*b*).

Some evidence in mice has suggested that foetal homologous tissues may cause less delayed immunological reaction than adult homologous tissues (Congdon and Urso, 1957). But Dixon and Weigle (1957) have recently shown that although baby rabbits cannot make antibodies, splenic and thymic cells from these animals injected into X-irradiated adult recipients are capable of agglutinin production. With these observations in mind it is worth noting that one of our animals treated with neonatal rabbit liver died at the 44th day with the syndrome of diarrhoea and wasting now associated with an immune reaction on the part of the introduced foreign tissues against the host (Uphoff, 1957 ; Trentin, 1957).

The proneness of rabbits to death from initial shock and gastric ulceration and perforation has been noted previously (Porter, 1957) and in the present experiments resulted in a high mortality despite successful haemopoietic transplantation.

SUMMARY

Injection of liver cells from baby female rabbits favourably modifies the post-irradiation course of male rabbits exposed previously to 900 r whole body X-irradiation. This is accompanied by persistence and function of donor haemopoietic cells, as shown by rapid haematological recovery and the appearance of female heterophils in the peripheral circulation of the irradiated host.

Post-irradiation treatment with neonatal mouse liver cells fails to exert any beneficial effect on irradiated rabbits. No evidence of cellular transplantation is obtained in this latter instance.

REFERENCES

Berenbaum, M. C.—(1956) *J. clin. Path.*, **9**, 381.
Congdon, C. C. and Urso, I. S.—(1957) *Amer. J. Path.*, **33**, 749.
Dixon, F. J. and Weigle, W. O.—(1957) *Fed. Proc.*, **16**, 411.
Ford, C. E., Hamerton, J. L., Barnes, D. W. H. and Loutit, J. F.—(1956) *Nature*, **177**, 452.
Gomori, G.—(1941) *J. cell. comp. Physiol.*, **17**, 71.
Jacobson, L. O.—(1952) *Cancer Res.*, **12**, 315.
Idem, Marks, E. K. and Gaston, E. O.—(1956*a*) *Radiation Res.*, **5**, 483 (Abstract).—(1956*b*) *Proc. Soc. exp. Biol., N.Y.*, **91**, 135.
Lindsley, D. L., Odell, T. T. and Tausche, F. G.—(1955) *Ibid.*, **90**, 512.
Lorenz, E., Uphoff, D., Reid, T. R. and Shelton, E.—(1951) *J. nat. Cancer Inst.*, **12**, 197.
Loutit, J. F.—(1954) *J. Nuclear Energy*, **1**, 87.
Makinodan, T.—(1956) *Proc. Soc. exp. Biol., N.Y.*, **92**, 174.
Merwin, R. M. and Congdon, C. C.—(1956) *Fed. Proc.*, **15**, 129.
Mitchison, N. A.—(1956) *Brit. J. exp. Path.*, **37**, 239.
Nowell, P. C., Cole, L. J., Habermeyer, J. G. and Roan, P. L.—(1956) *Cancer Res.*, **16**, 258.
Idem, Cole, L. J., Roan, P. L. and Habermeyer, J. G.—(1957) *J. nat. Cancer Inst.*, **18**, 127.
Porter, K. A.—(1957) *Brit. J. exp. Path.*, **38**, 401.
Trentin, J. J.—(1957) *Proc. Soc. exp. Biol., N.Y.*, **96**, 139.
Uphoff, D. E.—(1957) *J. nat. Cancer Inst.*, **19**, 123.
Vos, O., Davids, J. A. G., Weyzen, W. W. H. and Van Bekkum, D. W.—(1956) *Acta physiol. pharmacol. neerl.*, **4**, 482.

THE EFFECT OF METHYL CHOLANTHRENE ON GROWTH AND PREGNANCY

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IT has been shown previously (Jackson and Robson, 1957) that methyl cholanthrene will antagonise the action of a number of steroid hormones, including oestradiol and progesterone, when the carcinogen is applied locally to the target organ. The results strongly suggested that the carcinogen and hormones compete for some receptor in the cell and it seemed desirable therefore to investigate the effects of methyl cholanthrene on the growth of other tissues. In the present paper the action of this carcinogen on the growth of the kidney, and on pregnancy, are described.

METHODS

The experiments on pregnancy were performed on mice (albino, C strain) and rabbits, using the methods described by Didcock, Jackson and Robson (1956). In mice, conception was dated from the finding of the vaginal plug and in rabbits from the observed mating. The methyl cholanthrene was given in mice either by subcutaneous or intraperitoneal injection of a solution in olive oil, the dose in each case being administered in 0·1 ml. In rabbits, the drug was injected into the amniotic fluid as a suspension in normal saline. For this purpose the drug was ground to a very fine powder in a pestle and mortar before suspension in saline. The suspension was fine enough to be given through a 26 gauge needle. The volume in each case was 0·1 ml.

The effect of methyl cholanthrene on the growth of the kidney was investigated as follows. The drug was injected into the left kidney of day-old mice. Although an attempt was made to give these injections subcapsularly, this was not consistently achieved and in some cases the material went into the substance of the kidney. For injection the methyl cholanthrene was suspended in Tween saline. Again the drug was ground finely in a pestle and mortar and the requisite amount of saline with 0·01 per cent of Tween added. The Tween was used in order to keep the suspension in the dispersed state. The animals were anaesthetised by cooling, being placed in the refrigerator at about − 8° for 10–15 min. An incision was then made in the left subcostal region and the kidney exposed for injection. The volume of the injection was in all cases 0·01 ml. After the injection, no attempt was made to close up the peritoneum and the skin was sutured with silk. Any blood was carefully swabbed off and the animals were allowed to recover at room temperature, as it has been found that any artificial warming will increase the mortality. Only after full recovery are they replaced with the mother, and this usually takes between $\frac{1}{2}$ and $\frac{3}{4}$ hr. It is important to give the mothers an ample supply of water both before and after the operation. When all these precautions are observed, the mortality is of the order of 7 per cent. Some animals received a second intrarenal injection at 14 days of age, when the same precautions were observed, but ether was used as an anaesthetic.

RESULTS

Effects on pregnancy in mice

The results are shown in Table I. It will be seen that the effects are most striking when the drug is given at the time of the finding of the vaginal plug, *i.e.*, within 24 hr. of mating. In such animals the intraperitoneal injection of 1 mg.

TABLE I.—*Effect of Methyl Cholanthrene on Pregnancy in Mice*

Route of administration	Duration of pregnancy (days)	Dose of methyl cholanthrene (mg.)	Number of animals in which pregnancy was interrupted / Total number studied
Intraperitoneal	1	0·5	4/5†
	1	1·0	7/7†
	6–7	1·0	2/3
	11	1·0	0/3
Subcutaneous	1	2·0	3/5†
	1	5·0	11/11†
	6–7	1·0	3/5
	6–7	2·0	3/4
	6–7	5·0	6/6
	11	2·0	0/2
	11	5·0	0/4
Subcutaneous	7	2·0	2/5*
	7	5·0	7/7*

† A total of 50 mice, in which vaginal plugs were found, received no treatment. Thirty-nine of these animals became pregnant.

* Mice ovariectomised and received 1·5 mg. of progesterone per day.

resulted in no pregnancies developing and even 0·5 mg. had some effect. Subcutaneous administration of the drug was rather less effective, but a dose of 5 mg. per animal prevented development of pregnancy in every case. A laparotomy was performed on the 7th day in all animals that had received 1 mg. of methyl cholanthrene intraperitoneally and in every case no signs of pregnancy were found. Laparotomy was also performed on the 7th day on mice receiving 5 mg. per subcutaneous injection and in 5 of these small sites were present which, however, had disappeared at a second laparotomy 3 days later. The mice injected subcutaneously with 5 mg. of methyl cholanthrene on the 6th–7th day were examined by laparotomy on the 10th day. In 3 of them the uterus was empty and in 3 the pregnancy was in an advanced stage of reabsorption.

In order to determine whether the effect was due to the action of the drug on the ovary, ovariectomy was performed on the 7th day of pregnancy and 1·5 mg. of progesterone given from the previous day until the end of the experiment. This is ample to maintain pregnancy in ovariectomised mice (Robson, 1938). In such animals treated with 5 mg. of methyl cholanthrene subcutaneously, pregnancy was interrupted in all cases. As can be seen from Table I, a number of animals receiving methyl cholanthrene went to full term and gave birth to live litters. The average weight of the foetus in such litters was $1{\cdot}38 \pm 0{\cdot}18$ g. as compared with $1{\cdot}87 \pm 0{\cdot}24$ g. in the controls. These mice showed no congenital abnormalities and they were kept for 3 months and then examined post-mortem and no signs of tumour were found.

Five mg. of methyl cholanthrene was given intraperitoneally to 5 non-pregnant mice and no deleterious effects were observed up to one month, when they were killed for post-mortem examination.

Effects on pregnancy in rabbits

The results are shown in Table II. It will be seen that a dose of methyl cholanthrene of the order of 2–2·5 mg. consistently interrupted pregnancy. In all these cases reabsorption of the foetus was occurring within 5 days of the

TABLE II.—*Intra-amniotic Methyl Cholanthrene in Rabbits 16 Days Pregnant*

Dose of methyl cholanthrene in mg.	2·5	2·1	2·0	1·8	1·5	1·0
Number of foetal deaths/total number of sites injected	1/1	3/3	1/1	2/3	0/3	0/2

administration of the drug. In these animals, however, the weight of the placenta was not affected. For example, the placentae in 3 sites injected with 2·1 mg. of methyl cholanthrene weighed respectively 3·5, 4·15 and 3·1 g. (5 days after the injection when the foetuses had been practically completely reabsorbed), while in 13 control sites in the same animals the average weight of the placentae was 3·52 g.

The effect of methyl cholanthrene on growth of kidney

As all animals were injected into the left kidney with the carcinogen, this kidney being the easier to approach, it was first necessary to determine whether there are any differences between the weights of the 2 kidneys of a mouse at that particular age. Nineteen mice were killed at 4 weeks and the 2 kidneys removed, dissected free from fat and the pelvis cut off. They were weighed on a torsion balance. The average weights of the right and left kidneys were respectively 64·4 and 64·6 mg., and in only one case was there a difference of more than 3 mg. (actually 4·0 mg.) between the two sides.

In 12 mice the left kidney received 2 injections of methyl cholanthrene at 1 and 14 days of age. The kidneys were removed and weighed on the 28th day. The average weights of the left and right kidneys were 47·0 and 83·5 mg. respectively and in every case the left kidney weighed less than the right, the maximum difference being 76·0 mg. and the minimum 6·0 mg. There is thus no doubt that these injections produced a striking inhibition of kidney growth (Table III).

TABLE III.—*Effect of Methyl Cholanthrene on Kidney Growth*

Substance	Average weight of kidneys* (mg.) R.	Average weight of kidneys* (mg.) L.	Average weight of kidneys per 10 g. mouse (mg.) R.	Average weight of kidneys per 10 g. mouse (mg.) L.	Average body weight (g.)
No treatment .	64·6±2·69	64·4±2·57 .	92·3±1·5	92·0±1·68 .	7·0±0·66
Methyl cholanthrene	83·5±7·36	47·0±3·97 .	107·2±8·6	62·5±5·4 .	7·8±1·06
0·01 per cent Tween saline	62·6±3·41	58·6±3·15 .	91·3±3·34	85·5±3·12 .	6·9±0·4
Cholesterol . .	105·0±4·34	103·0±3·89 .	104·4±2·78	99·4±3·21 .	10·4±0·72
Sitosterol . .	110·4±2·99	107·6±2·12 .	83·1±1·98	81·0±1·76 .	13·3±0·93

* The left kidney was injected in all animals.

Histological appearances.—The kidneys injected with methyl cholanthrene showed thinning of the cortex and an apparent reduction in the number of glomeruli. No degenerative changes were seen in any of the sections.

It seemed possible that the mere injection of material into the kidney might depress its growth and, therefore, 3 control experiments were performed to check this point. In the first the suspending fluid, *i.e.*, 0·01 per cent Tween in saline, was injected in exactly the same way and at the same times into 14 mice which were also killed on the 28th day. The average weights of the left and right kidneys were 58·6 and 62·6 mg. respectively. In 4 animals the difference was more than 5

mg., the greatest difference being 12 mg. In the second control experiment a sample of cholesterol purified by re-crystallisation was used. It was tested for oestrogenic activity by local application to the vagina of ovariectomised mice in doses of 3 mg. The cholesterol was applied in powder form to the vagina which was then stitched up and the implant left in position for 7 days, before removing the vagina for histological examination. No oestrogenic effect was detected. The cholesterol was suspended in saline and injected into the kidney of 12 mice on the 1st and 14th days of life. Again the kidneys were removed on the 28th day and weighed. The average weights of the left and right kidneys were 103 and 105 mg. A similar experiment using purified sitosterol (previously tested for oestrogenic activity with negative results) gave average left and right kidney weights of 107·6 and 110·4 mg. (16 animals). It is clear from these control experiments that the effect of methyl cholanthrene was not merely due to the injection of material into the kidney. Histological examination of these kidneys also showed no abnormalities.

DISCUSSION

Methyl cholanthrene will interrupt pregnancy in mice when administered on the 1st or 7th days of gestation, whereas at mid-term it is comparatively ineffective. These actions are analogous to those of chromosomal poisons and antimetabolites.

The conceptus is more sensitive than the maternal tissues to the toxic action of this carcinogen, since none of the mothers died following methyl cholanthrene administration and a similar dose in non-pregnant animals was without apparent effect. The intra-amniotic injection of this carcinogen killed the foetus and left the placenta intact and, therefore, this substance can directly depress embryonic growth.

There is a considerable amount of evidence to suggest that methyl cholanthrene can affect pregnancy other than by a direct action on the embryo. Haddow and Robinson (1937) found that the carcinogenic hydrocarbons would depress ovulation in the rat and this, together with other observations, led them to suggest that the growth-inhibiting properties of these substances are, in part, the result of a depression of pituitary activity. Flaks (1948) has shown that methyl cholanthrene antagonises the effect of chorionic gonadotrophin on the ovary. However, ovariectomy with the administration of 1·5 mg. progesterone per day did not alter the effect of methyl cholanthrene and corpora lutea were found in the ovaries following interruption of pregnancy. It is unlikely, therefore, that methyl cholanthrene affects pregnancy either by a depression of pituitary gonadotrophin activity or by a direct effect on the ovary.

The experiments previously reported demonstrate that methyl cholanthrene will antagonise the action of steroid hormones on the growth of secondary sex organs and, therefore, this carcinogen may interfere with the steroid hormones responsible for the maintenance of pregnancy. This action of carcinogens requires further investigation before the exact mode of action of methyl cholanthrene can be established. However, the available evidence suggests that this is not the only mode of action, since the carcinogen has no effect at mid-term which might be expected if hormone antagonism was the only factor concerned. Experiments using doses of progesterone in excess of those required to maintain pregnancy in ovariectomised mice must be performed before the effect of methyl cholanthrene on pregnancy can be fully established.

Strong and Hollander (1947) considered the effect of methyl cholanthrene on pregnancy to be mediated through an effect on maternal metabolism since, with crosses of susceptible and resistant strains of mice, these workers found that the genetic structure of the mother, and not that of the embryo, was the dominant factor in determining the response to methyl cholanthrene. However, as only one dose level was used, it is possible that these results are due to a relative rather than an absolute resistance of certain strains.

The experiments also demonstrate that methyl cholanthrene will effectively depress kidney growth during the first month of life. The injection of 0·01 per cent Tween saline, cholesterol, or sitosterol did not appreciably affect the weight of the injected kidney and, therefore, the effect of methyl cholanthrene is the result of a specific action on growing tissue and not simply due to the injection of material into the kidney. In the carcinogen-treated animals there was no depression of body weight (see Table III) and, therefore, it is unlikely that much of the methyl cholanthrene was absorbed from the site of injection.

Histological examination of the kidneys showed that methyl cholanthrene produces thinning of the cortex with an associated weight reduction. In addition, no definite evidence of degenerative changes was found in the methyl cholanthrene injected kidneys, suggesting that the reduction in weight is due to a depression of growth as distinct from actual death of cells.

Arataki (1926) investigated kidney growth in the albino rat and showed that, during the first 100 days of life, the kidney grows by the formation of new glomeruli and tubules, the number being more than doubled in the first 25 days. This worker also compared the relative volume of cortex and medulla during the early stages of life and found that the percentage volume of the cortex in relation to total kidney size increases steadily from birth and reaches a maximum at about 35 days. When the total volume of glomeruli was compared with the size of the cortex, it was found that from birth to 20 days there is a rapid decrease in the percentage value of the volume of glomeruli in relation to the cortex. From this observation Arataki concludes that while the number of glomeruli is increasing, other structures in the kidney are growing much more rapidly. The overgrowth of these structures comes to an end at 90 days, which approximates to the time by which the adult number of glomeruli have been formed and suggests that the increase in size of the cortex is principally due to tubular growth as well as to the formation of new glomeruli.

On this basis, methyl cholanthrene would be expected to depress the growth of the cortex, the effect being most obvious in the growth of the tubules. The kidneys treated with methyl cholanthrene showed a reduction in the thickness of the cortex but on the evidence presented, it is not possible to decide which was principally affected.

The mode of action of methyl cholanthrene is unknown but there are 3 main possibilities. Firstly, the carcinogen could act as a chromosomal poison as described by Koller (1947) and, although no evidence of chromosomal fragmentation was seen in the sections, this possibility cannot be excluded in view of the high local concentrations of carcinogen employed. Secondly, in addition to this action, or as part of it, methyl cholanthrene could interfere with the utilisation of some specific factor in the cell. Thirdly, the drug could antagonise the action of specific growth factors on kidney growth and this would account for the depression of kidney growth without actual cell damage seen in these experiments. This requires

further investigation for, on the basis of the hypothesis of Robson and Keele (1956), it should be possible to antagonise the effect of methyl cholanthrene on kidney growth with large doses of the specific growth factor postulated by these workers.

SUMMARY

Methyl cholanthrene will interrupt pregnancy in mice when administered on the 1st or 7th days of gestation. The carcinogen will also directly depress embryonic growth in the rabbit. The mode of action is unknown but it is suggested that the effect is not mediated through the pituitary or ovary but is due to a direct effect on embryonic growth.

The injection of a suspension of methyl cholanthrene in Tween saline into the left kidney of new-born mice depresses the rate of growth of this organ during the first month of life. Pure cholesterol, sitosterol and Tween saline have no effect on kidney growth. It is concluded that the carcinogen acts by preventing the formation of new glomeruli and by depressing tubular growth. The possible mode of action is discussed.

We should like to thank the Medical Research Council for a grant (to J. M. R.) towards the expenses of the investigation; Mrs. D. Hills for dating the pregnancies and looking after the animals; and Mr. Drain of Messrs. Smith & Nephew Research Ltd., for the supply of cholesterol and sitosterol.

REFERENCES

ARATAKI, M.—(1926) *Amer. J. Anat.*, **36**, 399.
DIDCOCK, K. A., JACKSON, D. AND ROBSON, J. M.—(1956) *Brit. J. Pharmacol.*, **11**, 437.
FLAKS, J.—(1948) *Brit. J. Cancer*, **2**, 395.
HADDOW, A. AND ROBINSON, A. M.—(1937) *Proc. roy. Soc.*, B., **122**, 442.
JACKSON, D. AND ROBSON, J. M.—(1957) *J. Endocrin.*, **14**, 348.
KOLLER, P. C.—(1947) *Symp. Soc. exp. Biol.*, **1**, 270.
ROBSON, J. M.—(1938) *Quart. J. exp. Physiol.*, **28**, 195.
Idem AND KEELE, C. A.—(1956) In 'Recent Advances in Pharmacology', 2nd ed. London (Churchill), p. 483 ff.
STRONG, L. C. AND HOLLANDER, W. F.—(1947) *J. nat. Cancer Inst.*, **8**, 79.

THE PURIFICATION OF DIPHTHERIA TOXIN AND THE ISOLATION OF CRYSTALLINE TOXIN-PROTEIN

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ATTEMPTS to isolate the specific toxin produced by *Corynebacterium diphtheriae* have been made by many workers. Eaton (1936*a*, *b*), using culture filtrates produced with peptone-infusion medium, succeeded in isolating toxin with a purity of 0·0005 mg. nitrogen associated with 1 Lf of toxin, and Pappenheimer (1937), who used a medium of defined composition, isolated toxin of a similar purity. These workers were unable to increase the purity of their preparations by further fractionation. The immunochemical studies of Pappenheimer and Robinson (1937) and the physical-chemical studies of Pappenheimer, Lundren and Williams (1940) supported the view that the specific toxin had been isolated, and that 1 Lf unit of toxin was associated with 0·00046 mg. nitrogen. This figure was accepted for many years, but Pope, Stevens, Caspary and Fenton (1951) produced evidence to show that this value was incorrect. They employed methods similar to those of Derrien (1947), using potassium phosphate for the fractional precipitation of crude toxin, and showed that the protein precipitated in a series of exponential steps, with the Lf units of toxin associated with all the protein precipitated. By fractional precipitation in this way they isolated toxin with a purity in excess of that previously reported, and obtained, in small yield, a toxin fraction with a purity of 3200 Lf units per mg. nitrogen. This high value was confirmed when Pope and Stevens (1953*a*) reported the isolation of crystalline toxin-protein of this purity. Other workers have also obtained toxin with a purity considerably in excess of 0·00046 mg. nitrogen per Lf unit (see Pope, 1957).

This paper describes further work on the fractionation of diphtheria toxin with potassium phosphate, a new method for routine large scale purification of toxin, the isolation of the crystalline toxin-protein, and some of its properties.

MATERIALS AND METHODS

Diphtheria toxin.—For all the work reported here the Park-Williams No. 8 strain of *C. diphtheriae* was used. Initially, culture filtrates produced on tryptic or papain digest medium (80–100 Lf units per ml.), were concentrated by ultrafiltration to 4000–7000 Lf/ml. For experimental fractionation these were diluted, usually to 500 Lf/ml.

Later work was done with culture filtrates produced by growing *C. diphtheriae* in submerged culture, Linggood (1953) and Linggood, Matthews, Pinfield, Pope and Sharland (1955); these filtrates with an initial value of 250–300 u./ml. and a high purity (ca. 1500 Lf units/mg. protein nitrogen) made ultrafiltration unnecessary for the purification method described later.

All culture filtrates were preserved by the addition of 0·01 per cent (w/v) Thiomersalate (B.D.H.) followed by sterile filtration through a press (British Filters Ltd.).

Ultrafiltration.—This was based on the method of Wadsworth and Quigley (1934) using a membrane of 8 per cent (w/v) collodion in acetic acid, deposited on Berkefeld candles. A battery of 12 candles was employed together with slow speed stirring and negative pressure to draw the ultrafiltrate through the candles.

Charcoal.—Most of the work reported here has been done with a steam-activated charcoal (Farnell's LS grade) although it has been found experimentally that other types of charcoal may be employed.

Total nitrogen.—Determinations were carried out as described by Pope and Stevens (1953*b*).

Protein nitrogen.—Precipitation of the protein was done in tapered centrifuge tubes by adding trichloracetic acid to 5 per cent followed by warming to 40° until good flocculation of the protein had occurred. After centrifuging, the precipitate was washed twice with 2 per cent (w/v) trichloracetic acid and the nitrogen content of the precipitate was determined.

Flocculation tests.—Antitoxin standardized against the International Flocculation Standard antitoxin was used for all this work.

Gel-diffusion tests.—Where the diffusion test was done in tubes, the method described by Oakley and Fulthorpe (1953) was used except that gelatin was substituted for agar and diffusion was alowed to proceed at 2–3° (Pope *et al.*, 1951). Plate diffusion tests using agar as described by Ouchterlony (1948) have also been used for some experiments.

EXPERIMENTAL

Fractionation with Phosphate

Pope, Stevens, Caspary and Fenton (1951) using the phosphate precipitation method of Derrien (1947) found that in all the preparations of diphtheria toxin examined both the protein and the toxin were precipitated in a series of exponential steps. The toxin, as measured by the flocculation test, was associated with all the protein precipitated, and no clear-cut separation could be obtained. Some degree of purification was possible using phosphate mixture at pH 7·4 but the loss of Lf units with the early protein fractions reduced the yield of high purity toxin.

Consideration of these results suggested that the different proteins present might differ in their isoelectric points, and that with the precipitation of the least soluble proteins, the formation of a solid phase could lead to the adsorption and co-precipitation of more soluble proteins with a higher isoelectric point. It was, therefore, decided to investigate the precipitation of toxin using phosphate mixtures at different pH values, on the assumption that the specific toxin might be less readily adsorbed on other proteins at higher pH values. Before doing this it was necessary to determine the stability of the specific toxin in phosphate mixtures at these pH values.

Using potassium phosphate solutions of varying strengths in the pH range 7 to 10 the stability of the specific toxin was determined in terms of Lf units/ml., Lr units/ml., and minimal reacting doses (M.R.D.)/ml. In this way any change in either the *in vitro* or *in vivo* properties of the toxin gave an indication of damage or denaturation of the toxin at the higher pH values. No real evidence of damage to the toxin was found within this pH range for periods of exposure up to 48 hours at 15–20°. This made it possible to repeat the work of Pope *et al.* (1951) using alkaline phosphate mixtures for the toxin precipitation by Derrien's method. A systematic study showed that with increasing pH values towards the upper limit, protein practically free of specific toxin was first precipitated, and that at pH 10 the separation of the bulk of the non-specific protein could be achieved for the loss of 10 per cent of the original Lf units. The remaining toxin precipitated

in what appeared to be a single exponential step with a purity exceeding 2000 Lf/mg. protein nitrogen and a yield of 85–90 per cent.

The ability to work at high pH values made it possible to use solid K_2HPO_4 for the precipitation, and to add this until control tests showed that about 10 per cent of the toxin had precipitated. Filtration, followed by the addition of further solid K_2HPO_4 to the filtrate, to precipitate the purified toxin, gave satisfactory results. Material isolated in this way could with advantage be refractionated with solid K_2HPO_4 to give products of increased purity with little additional loss of Lf units. When examined by gel-diffusion methods these purified toxins showed one major line with several minor lines.

Refractionation in this way using solid K_2HPO_4 resulted in the first isolation of crystalline toxin-protein reported by Pope and Stevens (1953*a*); other methods now used are given later.

Ammonium Sulphate-Charcoal Process

The need of a method for routine large-scale purification of diphtheria toxin or toxoid prepared on either tryptic or papain digest medium led to a very extensive investigation of the problem. It was desirable that it should be simple to operate and give high yields of toxin with a purity exceeding 2000 Lf units/mg. protein nitrogen. Quite early in this work it was realized that it was easier to purify toxin rather than the toxoid prepared from it. Differences which existed in the physical and chemical properties of proteins present in the crude culture filtrates tended to disappear after they had been modified by the action of formaldehyde. It was also found that diphtheria toxin concentrated by ultrafiltration to 500 to 1000 Lf/ml. was a more suitable material for fractionation procedures than the crude filtrate. This presented no difficulty as the ultrafiltration was mechanised and required little labour for its operation.

Schmidt and Hansen (1930), Pope and Linggood (1939) and Linggood (1941) used charcoal in the purification of toxoid. Unfortunately charcoal varies very considerably in its properties and these later workers found only one brand of charcoal, " Eponit " C, that had a markedly selective action in removing unwanted protein and increasing the specific activity of the toxin or toxoid.

Experimental work carried out in an attempt to find the reason for the variable behaviour of charcoal led eventually to the method described here. It has been in routine operation for many years and has always given satisfactory results. This method depends on the use of $NaHCO_3$ 0·5 per cent (w/v) in order to keep the pH on the alkaline side, and the employment of charcoal under conditions where " forced adsorption " takes place by carrying out the adsorption in the presence of 25 per cent (w/v) ammonium sulphate.

In this method it is essential to dissolve the $NaHCO_3$ in the toxin solution before adding the ammonium sulphate and charcoal, otherwise most of the specific toxin will be adsorbed on the charcoal and permanently destroyed.

The optimal amounts of $NaHCO_3$ and $(NH_4)_2SO_4$ have been determined as the result of experimental work on many batches of toxin. Only the concentration of the charcoal must be determined for the particular sample of toxin being purified. A steam-activated charcoal (Farnell's LS grade) is usually employed, but experimental work has shown that charcoals prepared by other activation methods (phosphoric acid or zinc chloride) are also suitable although they may be a little more active on a weight basis than the steam-activated charcoal.

This is of little consequence as the actual amount of charcoal must be determined; a slight preference for steam-activated charcoal is based on its lower activity which simplifies control in a routine process. Under the conditions of "forced adsorption" used here the amounts of charcoal required are less than those employed by Pope and Linggood (1939) or Linggood (1941).

The determination of the correct amount of charcoal is carried out using a portion of the main batch of toxin, diluted to about 500 Lf/ml., and containing 0·5 per cent (w/v) $NaHCO_3$ and 25 per cent (w/v) ammonium sulphate. To 50 ml. portions a suitable range of charcoal concentrations is added with the object of determining the amount of charcoal that leaves about 70 per cent of the original Lf in the filtrate. When toxin partially purified by ultrafiltration and of good initial purity is used, the amount of charcoal may be reduced to obtain a higher yield (see Table I). After adding the charcoal and mixing, a period of 1 hr. is allowed before filtration. Usually only a small amount of precipitation is obtained by the addition of 25 per cent (w/v) ammonium sulphate with virtually no loss of Lf units. There is approximately a 10 per cent increase in volume after adding the ammonium sulphate which must be allowed for in determining the Lf values.

TABLE I.—*Purification of Ultrafiltered Diphtheria Toxins JG* 188 *and RX* 4994 *C*

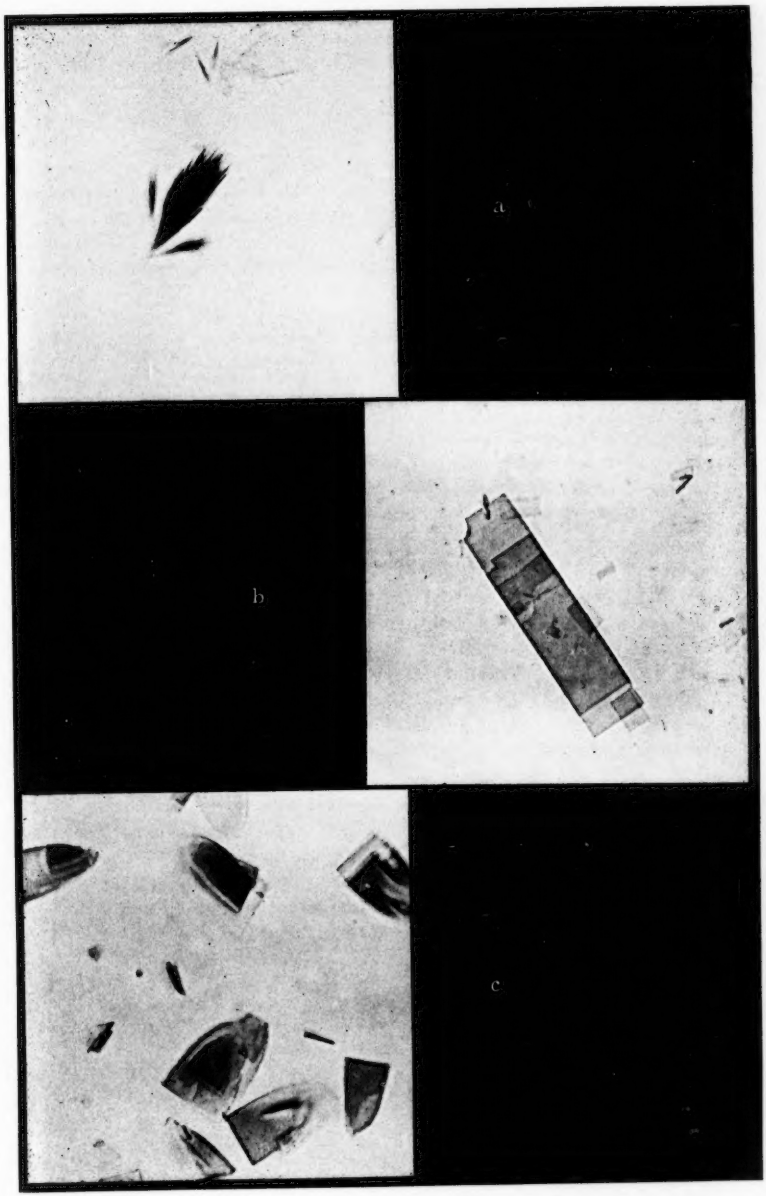
Procedure, details etc.	Stage No.	Volume litres	Lf u./ml.	Lf yield
Ultrafiltered toxin	1	17·8 (8·0)	1690 (4670)	100 (100)
Diluted to 500 u./ml. and 0·5 per cent w/v $NaHCO_3$ added	2	60·2 (71·2)	520 (535)	100 (100)
$(NH_4)_2SO_4$ 25 per cent w/v, Charcoal see below w/v } added.				
Filtered on Büchner filter using Hyflo bed Filtrate	3	66·2 (77·8)	367 (412)	77 (84)
Per cent $(NH_4)_2SO_4$ w/v added to filtrate (see blow). Allowed to stand overnight, clear supernatant decanted and precipitate collected on Büchner filter with thin Hyflo bed	4	—	—	—
Precipitate washed with $(NH_4)_2SO_4$ solution containing 0·5 per cent $NaHCO_3$ (see below)	5	—	—	—
Precipitate dissolved in 0·1 per cent w/v $NaHCO_3$ (see below)	6	6·0 (7·56)	3970 (4070)	76 (80)
Toxin dialysed in cellophane bags for 72 hr. until sulphate-free. Contents of bags pooled and washings from bags added	7	12·0 (14·0)	1860 (2100)	72 (77)

JG 188: Original purity 1760 Lf/mg. P.N. Final purity 2720 Lf/mg. P.N.
RX 4994 C: All figures in brackets in Table I.
Original purity 2000 Lf/mg. P.N. Final purity 2625 Lf/mg. P.N.
Stage 3: The amount of charcoal used was 0·9 per cent w/v for JG 188 and 0·7 per cent w/v for RX 4994 C.
Stage 4: $(NH_4)_2SO_4$ added was 17 per cent w/v for JG 188 and 19 per cent w/v for RX 4994 C.
Stage 5: For JG 188 6 litres of 44 per cent w/v $(NH_4)_2SO_4$ with 0·5 per cent $NaHCO_3$.
For RX 4994 C 4 litres of 46 per cent w/v $(NH_4)_2SO_4$ with 0·5 per cent $NaHCO_3$.
Stage 6: The toxin was extracted from the filter cake with successive lots of 0·1 per cent $NaHCO_3$ solution.

EXPLANATION OF PLATE.

FIG.—Crystalline diphtheria toxin-protein showing three types of crystals obtained:

(*a*) Once crystallized from K_2HPO_4;
(*b*) recrystallized from K_2HPO_4;
(*c*) crystallized from $(NH_4)_2SO_4$.



Pope and Stevens.

A loss of about 30 per cent of the original Lf units by adsorption on the charcoal is practically the only loss involved in the whole process.

The toxin present in the filtrate from the charcoal stage is precipitated by the addition of more ammonium sulphate, usually to 44 per cent (w/v), collected by filtration, redissolved in suitable volume, and dialysed until free of ammonium sulphate.

Table I gives the details of routine batches purified in this way. In both cases the initial purity of the ultrafiltered toxin was good. The yields at stage 3 were 77 and 84 per cent respectively with the smaller amount of charcoal used, and the overall yields 72 and 77 per cent with final purities of 2720 and 2625 Lf/mg. protein nitrogen. These results indicate the flexibility and value of this method.

With the introduction of submerged culture methods for the production of *C. diphtheriae* toxin (Linggood *et al.*, 1955), filtrates containing 200–300 Lf units/ml. became available for purification, and with some modification of the method the need for ultrafiltration was avoided. To the crude toxin filtrate is added 0·5 per cent $NaHCO_3$ followed by 25 per cent (w/v) $(NH_4)_2SO_4$; the amount of charcoal required to give about 70 per cent Lf yield in the filtrate is then determined; it is in the range 2–4 per cent (w/v) but for less pure toxin filtrates may be as high as 5 per cent (w/v). The toxin is precipitated from the filtrate by the addition of more $(NH_4)_2SO_4$ to 44 per cent (w/v), separated by filtration, dissolved and dialysed as for ultrafiltrated toxin.

Further Purification of the Charcoal-sulphate Concentrate

Two methods have been used for the further fractionation of the purified concentrates. In the first method K_2HPO_4 was used as the precipitating agent. The aim was to approach the initial precipitation point cautiously in order to remove protein carrying very little toxin with it, and then to precipitate the bulk of the toxin, with the minimum concentration of precipitant, so that any non-toxin proteins remained in the waste filtrate. Table II illustrates a fractional separation with K_2HPO_4. Surface culture toxin after ultrafiltration was purified to 2440 Lf units/mg.protein nitrogen by means of the charcoal-ammonium sulphate process; for use it was diluted to 500 u./ml. Three major fractions of increased purity, totalling 79 per cent of the starting Lf value, were obtained; the third fraction crystallized in needle form and gave the first indication that at sufficiently high purity the toxin-protein could be crystallised. Table III shows another fractionation by this method. Comparison of Tables II and III shows that the major portion of the toxin is precipitated between the limits 35–39 per cent (w/v) K_2HPO_4 with a definite increase in purity.

TABLE II.—*Fractionation of Purified Diphtheria Toxin with Solid K_2HPO_4*

Fraction No.	Per cent K_2HPO_4 (w/v)	Per cent Lf yield		Lf/mg. P.N.
1	0–35	26·5		2500
2	35–37	28·5	79	2640
3	37–39	24·0		2760
4	39–41	3·0		Not done.
Waste filtrate and washings*	—	12·5		—

* Each precipitate was washed with a little phosphate solution of the strength used for precipitation.

Original purity after charcoal-ammonium sulphate process 2440 Lf/mg. P.N.

TABLE III.—*Fractionation of Purified Diphtheria Toxin with Solid K_2HPO_4*

Fraction No.	Per cent K_2HPO_4	Per cent Lf yield	Lf/mg. P.N.
1	0–30		
2	30–33	5	Not done.
3	33–35		
4	35–39	90	2800*
Waste filtrate	At 39	2	—

* Original purity after charcoal-ammonium sulphate process 2530 Lf/mg. P.N.

Some difficulty has been experienced in obtaining good crystallization with certain batches of toxin but in the light of recent work it is considered that this is due to the length of time between preparation of the culture filtrates and subsequent stages of purification. With the use of fresh culture filtrates of high Lf value and high original purity (Linggood *et al.*, 1955) produced by submerged culture methods, these variation have become less obvious.

After the isolation of the crystalline toxin-protein as a result of phosphate fractionation (Pope and Stevens, 1953*a*), experiments were done to determine whether other precipitants could be used instead of phosphate. Both trisodium citrate and ammonium sulphate (used in the presence of $NaHCO_3$) gave crystalline toxin-protein preparations. Table IV shows results obtained using surface culture, ultrafiltered material, purified by the ammonium sulphate charcoal method, and then refractionated with ammonium sulphate. Contamination with amorphous material was avoided in later work by the use of fresh submerged-culture crude toxin filtrates.

TABLE IV.—*Fractionation and Crystallization of Diphtheria Toxin Previously Purified by the Ammonium Sulphate-Charcoal Method*

Fraction	Per cent Lf yield	Purity Lf/mg. P.N.	Precipitate form
Charcoal-sulphate purified	100	—	—
$(NH_4)_2SO_4$ (w/v)—			
25–28 per cent	0·5	—	Not crystalline.
28–31 ,, ,,	0·5	—	,, ,,
31–34* ,, ,,	42	2800	Crystalline (*a*).
34–36 ,, ,,	42	3050	,, (*c*).
36–40 ,, ,,	9	2470	Amorphous with few crystals.
Waste filtrate	4	—	—
Total	98		

* 31–34 per cent fraction, dialysed and refractionated as below—

31–34 per cent dialysed	100	2800	—
0–32 per cent	2	—	—
32–33 ,, ,,	59	3140	Crystalline (*a*)
33–36 ,, ,,	23	3000	,,
36–40 ,, ,,	5	3030	Crystalline and some amorphous
Waste filtrate	1·5	—	—
Total	90·5		

(*a*), (*c*) see Fig. 1.

Crystallization of Toxin-protein

Toxin, purified by the charcoal-ammonium sulphate method, is crystallized as follows :

To the toxin, diluted to about 500 Lf/ml., is added 0·5 per cent (w/v) $NaHCO_3$ followed by solid $(NH_4)_2SO_4$ to a concentration of 30 per cent (w/v). After standing overnight a precipitate forms, which is dark in colour and often gelatinous. This is removed by filtration on a Büchner filter with a Hyflo bed. Little loss of Lf units occurs at this stage. Where ultrafiltered toxin has been employed this filtrate is usually slightly coloured; when fresh submerged culture toxin is purified this filtrate is colourless. The $(NH_4)_2SO_4$ concentration in the filtrate is increased by 2 per cent (w/v), and crystallization usually starts in a few hours. After standing overnight at 16° a further 2 per cent (w/v) $(NH_4)_2SO_4$ is added. Crystallization is aided by stirring with a glass rod, care being taken to avoid frothing. At this concentration (34 per cent (w/v) $(NH_4)_2SO_4$) all but about 10 per cent of the toxin is precipitated in crystalline form (total time 48 hr.).

In some cases the toxin behaves rather differently and at 32 per cent (w/v) $(NH_4)_2SO_4$ no precipitate forms ; it is then necessary to increase the $(NH_4)_2SO_4$ to 34 per cent and subsequently to 36 per cent to get maximum crystallization.

The crystalline material is collected on a Büchner filter with a Hyflo bed and the cake of crystalline material is washed with a 0·5 per cent $NaHCO_3$ solution containing $(NH_4)_2SO_4$ 2 per cent (w/v) stronger than the final $(NH_4)_2SO_4$ concentration used for crystallization. The crystalline toxin-protein is re-dissolved using 0·5 per cent (w/v) $NaHCO_3$ and may be dialysed free of ammonium sulphate, or recrystallized, as desired.

Up to ten recrystallizations have been carried out using the folowing method. Crystalline toxin-protein is diluted to 500 to 1000 Lf units/ml., $NaHCO_3$ 0·5 per cent (w/v) added and then $(NH_4)_2SO_4$ to 28 per cent (w/v). In recrystallizations the toxin usually commences to crystallize at this lower sulphate level and may be completed by slow additions of more $(NH_4)_2SO_4$ to a final level of 34 per cent. Dialysis between the successive crystallizations is not necessary.

Several years experience with a large number of different toxin preparations has shown that Lf yield in the crystalline toxin-protein should not be less than 50 per cent of the Lf units present in the initial crude culture filtrate, *i.e.*, the total loss, in both the charcoal-ammonium sulphate and crystallization stages, is less than 50 per cent.

The crystallization of the toxin-protein does not appear to be influenced by seeding with crystals, and is not very temperature-sensitive ; generally, room temperature (16°) has been found satisfactory. Occasionally a batch of toxin fails to crystallize or gives a mixture of crystalline and amorphous material. This is probably due to the use of old toxin preparations ; there does not appear to be any way of inducing the amorphous material to crystallize. Crystalline toxin-protein after conversion to toxoid by means of formaldehyde also fails to crystallize and it is possible that any toxoid produced by natural means, such as ageing, might prevent crystallization.

Properties of the Crystalline Toxin-protein

In the course of this work the toxin-protein has been isolated in different forms. The Figure shows three forms of crystals. The needle forms were obtained when the initial crystallization was carried out using K_2HPO_4 but on recrystallization with K_2HPO_4 the plate form was obtained on several occasions. The shield type of crystal is more commonly obtained using $(NH_4)_2SO_4$ for crystallization from submerged culture filtrates from papain digest medium. No obvious differences in the properties of these forms have been found.

The use of Thiomersalate as the antiseptic in the crude culture filtrates suggested the possibility that mercury might play some part in the crystallization but this was disproved by using toluol as the preservative.

In the diffusion test described by Oakley and Fulthorpe (1953) (as modified by Pope *et al.*, 1951, who substituted gelatin for agar) the once crystallized material gave a major line and several minor ones. After several recrystallizations it was possible to obtain a product which gave a single line when tested at a toxin level of 2000 Lf/ml. against antitoxin at 2000 units/ml.

The question of the identity of the specific toxin with the crystalline toxin-protein is of considerable importance. Pope and Stevens (1953*a*) expressed doubt because of anomalous results with different antitoxins and Pope (1954) produced evidence to show that after heating crystalline toxin-protein at 100° for 16 min., the quantitative precipitation between the heated toxin and antitoxin was little affected although the ability of the heated toxin to neutralize the specific antitoxin (*in vivo* tests) had been reduced to about 5 per cent of its original value. Additional evidence has been given (Pope, 1957) in support of the view that the crystalline toxin-protein contains a series of antigens each capable of producing a specific antibody and that the specific toxin, responsible for the lethal and dermonecrotic properties, is only a part of the complex. Much additional work has been done which forms the subject of another paper (Pope and Stevens, 1958—in preparation).

The physical-chemical studies of Pappenheimer, Lundgren and Williams (1940) suggested that their toxin preparation of 2170 Lf/mg. nitrogen (less pure than the crystalline toxin-protein) was electrophoretically homogeneous. However, Lepow and Pillemer (1952) disagreed with these findings and suggested a value of 2600 Lf/mg. nitrogen as a minimum one for electrophorectially pure toxin.

A considerable amount of work has been done (Pope—unpublished) using paper electrophoresis, with a series of buffers of varying pH and ionic concentrations. In all cases the crystalline toxin-protein moved as a symmetrical component whether measured in terms of flocculating units or animal values (Lr or M.R.D.). There was always close agreement between *in vitro* and *in vivo* tests and no indication of separation. However, Poulik (personal communication) using starch-gel electrophoresis and other buffer solutions has obtained separation of the components in a crystalline toxin-protein prepared by us.

Katsura, Kato, Nakamura and Kayama (1957) examined toxins with purities between 2000 and 2500 Lf/mg. nitrogen and found none that gave patterns satisfying the usual criteria of electrophoretic homogeneity. Zone electrophoresis in starch-supported media gave them material with 3200 to 3300 Lf/mg. nitrogen, and at this purity the electrophoretic pattern indicated that the material was now homogeneous. These workers have also isolated from their highly purified toxin a crystalline toxin-protein with a purity of 3200–3300 Lf/mg. nitrogen thus confirming the work of Pope and Stevens (1953*a*).

The crystalline toxin-protein reacts with formaldehyde to give toxoid which is suitable for immunization procedures in man and animals. When used for hyperimmunization in the horse it was found that the resulting antibody serum gave only one line in the gel-diffusion test against crude toxin, even when tested at high unit levels. In further tests of the antitoxin it was found that it agglutinated washed suspensions of *C. diphtheriae*. The antibody responsible was easily

removed by absorption with *C. diphtheriae* without loss of the specific antitoxin. Thus, notwithstanding the very high purity of the crystalline-material used for the hyperimmunization of the horse, some antigen capable of stimulating the production of agglutinins for *C. diphtheriae* must have been present. Its removal by absorption without any loss of specific antitoxin indicates that it was a distinct antibody unrelated to the specific antitoxin; its presence shows how difficult it is to achieve immunological purity in an antigen.

Some preliminary work on the chemical composition of the crystalline toxin-protein was abandoned when it was realized that the crystalline material was a complex or mixture of antigens with distinct immunological properties, and that, as shown by Pope (1957), there was doubt about the amount of specific toxin present in these crystalline preparations.

DISCUSSION

The methods given for the purification and subsequent crystallization of the toxin-protein of *C. diphtheriae* are the outcome of extensive work over a number of years which it is not possible to describe here in detail. The following are the important factors which have led to these advances :—

Fractionation of diphtheria toxin at high pH values using either K_2HPO_4 or a combination of $NaHCO_3$ and $(NH_4)_2SO_4$ to avoid the co-precipitation of the toxin with the less soluble unspecific protein.

The use of charcoal in combination with $NaHCO_3$ and $(NH_4)_2SO_4$ in order to obtain a " forced adsorption " on the charcoal of the unspecific proteins.

Refractionation of highly purified toxin by means of K_2HPO_4 or $NaHCO_3$–$(NH_4)_2SO_4$ to obtain a crystalline product.

By means of these methods, overall yields greater than 50 per cent of the Lf units present in the original crude culture filtrate may be obtained as crystalline material.

When these methods are considered in conjunction with modern methods of toxin production (Linggood *et al.*, 1955), by which it is possible to produce culture filtrates containing 250–300 Lf in 47 hours, it is clear that there is no difficulty in producing large amounts of highly purified toxin.

The crystalline toxin shows an *in vivo/in vitro* ratio of 1 which indicates that the toxin has not been damaged by the purification methods; also, that any natural toxoid has been eliminated during the purification, since this ratio is better than that of the crude filtrates. The determination of the number of minimal reacting doses per Lf unit using the guinea-pig shows that 1 Lf unit contains about 50,000 M.R.D. This figure is higher than that quoted by Katsura *et al.* (1957) who found 1 Lf unit contained 40,000–50,000 M.R.D. as measured in the rabbit, a more sensitive animal.

The use of freshly prepared toxin filtrates for both the initial purification and subsequent crystallization is advantageous; this applies particularly to the crystallization stage because of the importance of avoiding contamination of the crystalline material with amorphous precipitate. While most of the work has been done with either tryptic or papain digest medium, the ammonium sulphate-charcoal method (but not the subsequent crystallization) has been applied to

casein hydrolysate culture filtrates with success. Too little of this type of culture filtrate was available, as freshly prepared material, for a complete study to the crystallization stage. However, Norlin (private communication) has obtained crystalline toxin-protein from casein hydrolysate culture filtrates. Katsura *et al.* (1957) used a medium based on casein hydrolysate as described by Yoneda (1951) and they also isolated crystalline toxin-protein. There is evidence therefore, that isolation of the crystalline toxin-protein is not confined to culture filtrates produced on digest medium.

The properties of the crystalline toxin-protein are very conflicting; by paper electrophoresis the toxin-protein appears to be a homogeneous material. The unpublished work of Poulik, does however, suggest that this is not necessarily true. The immunological results of Pope (1954, 1957) and the observations of Ambrose and Easty (1954) together with additional work (Pope and Stevens—in preparation) indicate very strongly that antigens with different properties are present in the crystalline material. These properties are not modified by repeated recrystallization of the toxin-protein. No suggestion that the crystalline toxin-protein and the specific toxin responsible for the lethal and dermonecrotic effects are identical is made in this paper.

SUMMARY

Factors affecting the purification of diphtheria toxin have been examined by carrying out the fractionation at high pH values using either K_2HPO_4 or an $(NH_4)_2SO_4$–$NaHCO_3$ system the adsorption and co-precipitation of toxin with the unspecific protein is avoided.

Are investigation of the properties of charcoal for toxin purification has provided a method whereby the unspecific proteins are removed by "forced adsorption" on the charcoal in an $(NH_4)_2SO_4$–$NaHCO_3$ system.

The further purification and crystallization of the toxin-protein is described. In these methods the primary purification requires the determination of only one variable, that is, the amount of charcoal necessary. Yields of crystalline toxin-protein exceeding 50 per cent of the original Lf units are readily obtained.

Some properties of the crystalline toxin-protein are considered.

Since this paper was prepared it has been found that Tiselius (1948) reported that increasing concentrations of $(NH_4)_2SO_4$ or potassium phosphate caused increased adsorption of protein on filter paper or silica gel. Hagdahl and Tiselius (1952) used the term 'salting-out' adsorption for this effect. We were not aware of these observations when our work was done.

Our thanks are due to Mr. E. A. Jones for the photographs of the crystalline toxin-protein.

The Farnell LS grade charcoal was obtained from Farnell Carbons Ltd., 1A Conduit Street, Plumstead, London.

REFERENCES

Ambrose, E. T. and Easty, G. C.—(1954) *Disc. Faraday Soc.*, **18**, 364.
Derrien, Y.—(1947) *Särtryck ur Svensk Kemisk Tidekrift*, **59**, 139.
Eaton, M. D.—(1936*a*) *J. Bact.*, **31**, 347.—(1936*b*) *Ibid.*, **31**, 367.

HAGDAHL, L. AND TISELIUS, A.—(1952) *Nature*, **170**, 799.
KATSURA, T., KATO, I., NAKAMURA, H. AND KOYAMA, J.—(1957) *Jap. J. Microbiol.*, **1**, 213.
LEPOW, I. H. AND PILLEMER, L.—(1952) *J. Immunol.*, **69**, 1.
LINGGOOD, F. V.—(1941) *Brit. J. exp. Path.*, **22**, 255.—(1953) 6th Int. Congr. Microbiol., Rome.
Idem, MATTHEWS, A. C., PINFIELD, S., POPE, C. G. AND SHARLAND, T. R.—(1955) *Nature*, **176**, 1128.
OAKLEY, C. L. AND FULTHORPE, A. J.—(1953) *J. Path. Bact.*, **65**, 49.
OUCHTERLONY, O.—(1948) *Acta Path. microbiol. scand.*, **25**, 186.
PAPPENHEIMER, A. M.—(1937) *J. biol. Chem.*, **120**, 543.
Idem, LUNDGREN, H. P. AND WILLIAMS, J. W.—(1940) *J. exp. Med.*, **71**, 247.
Idem AND ROBINSON, E. S.—(1937) *J. Immunol.*, **32**, 291.
POPE, C. G.—(1954) *Disc. Faraday Soc.*, **18**, 323.—(1957) *Brit. J. exp. Path.*, **38**, 207.
Idem AND LINGGOOD, F. V.—(1939) *Brit. J. exp. Path.*, **20**, 297.
Idem AND STEVENS, M. F.—(1953*a*) *Lancet*, ii, 1190.—(1953*b*) *Brit. J. exp. Path.*, **34**, 56.—(1958) *Ibid.* (in press).
Iidem, CASPARY, E. A. AND FENTON, E. L.—(1951) *Ibid.*, **32**, 246.
SCHMIDT, S. AND HANSEN, A.—(1930) *Biochem. Z.*, **228**, 263.
TISELIUS, A.—(1948) *Ark. Kemi*, **26B**, 1.
WADSWORTH, A. AND QUIGLEY, J. J.—(1934) *Amer. J. Hyg.*, **20**, 225.
YONEDA, M.—(1957) *Brit. J. exp. Path.*, **38**, 190.

THE PROPERTIES OF CRYSTALLINE DIPHTHERIA TOXIN-PROTEIN: ITS ANTIGEN COMPOSITION AS DETERMINED BY GEL-DIFFUSION METHODS

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THE isolation of a crystalline protein with both the *in vivo* and *in vitro* properties of diphtheria toxin from culture filtrates of *Corynebacterium diphtheriae* was reported by Pope and Stevens (1953*a*) who expressed doubt regarding the identity of the specific toxin with the crystalline protein they had isolated.

Pope (1954) showed that after heating to 100° for 16 min., the crystalline toxin-protein had lost most of its property of combining with and neutralizing the specific antitoxin (*in vivo* tests), yet it still gave almost as much floccule precipitate when mixed with antitoxic serum as did the unheated toxin. Further evidence for the heterogeneity of the crystalline toxin-protein was given by Pope (1957).

This paper describes the methods that have been used to examine the problem of the antigenic complexity of the crystalline toxin-protein using gel-diffusion methods.

EXPERIMENTAL

The change from "flocculating" to "precipitin" type of reaction in the diphtheria toxin-antitoxin system was reported by Pope (1954). Although crystalline toxin-protein was employed the flocculation curve was irregular and the amount of precipitate formed was less than in the "precipitin" system. It was suggested that the irregular flocculation curve indicated a series of antigen-antibody reactions all taking place in the flocculation zone. For each antigen-antibody

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EXPLANATION OF PLATES.

FIG. 1.—The action of 1 per cent K_3PO_4 on crystalline toxin-protein (2 hr. at 20°). Upper reservoir—peptic refined antitoxin 500 u/ml. Lower left—3 × crystalline toxin-protein. Lower right—same toxin after K_3PO_4 treatment.

FIG. 3.—Crystalline toxin-protein treated with 1 per cent tetra-sodium-versenate (2 hr. at 20°). Upper reservoir—peptic-refined antitoxin 500 u/ml. Lower left—crystalline toxin-protein. Lower right—versene treated toxin.

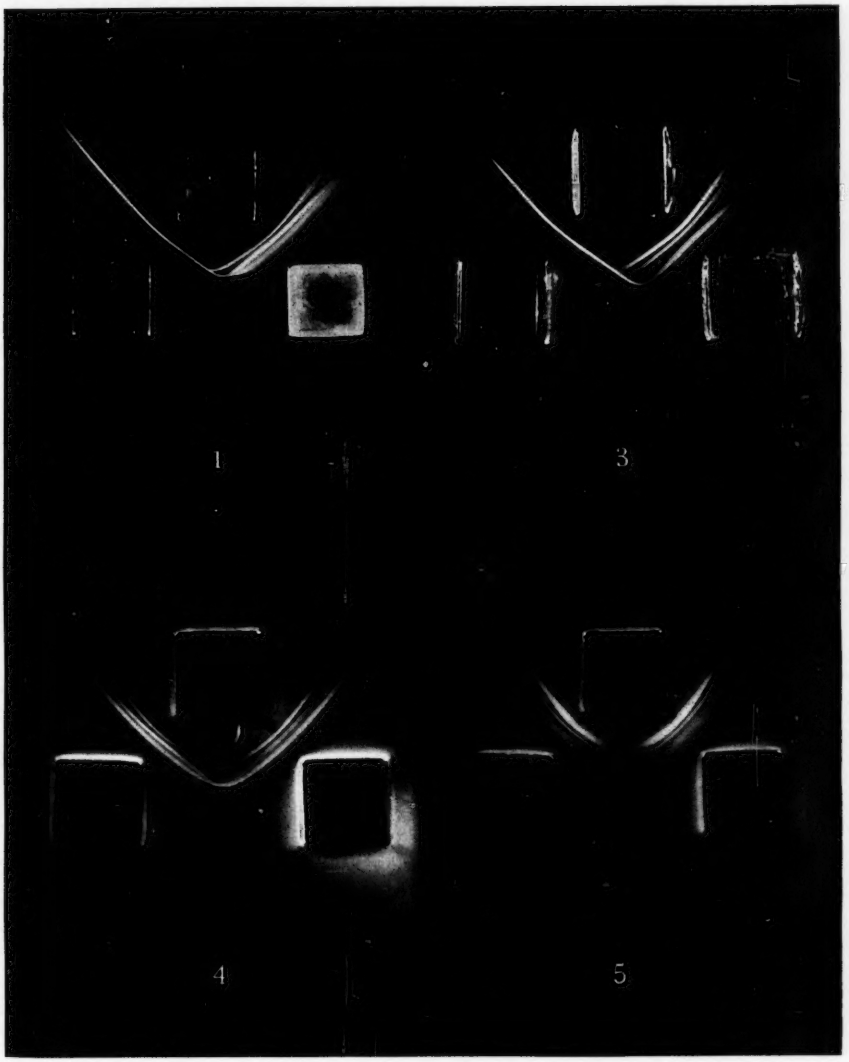
FIG. 4.—Upper reservoir—peptic-refined antitoxin 500 u/ml. Lower left—crystalline toxin-protein after 2 days' digestion with trypsin. Lower right—same after 3 days' digestion.

FIG. 5.—The digestion of crystalline toxin-protein with pepsin. Upper reservoir—peptic-refined antitoxin 500 u/ml. Lower right and left—pepsin digested toxin.

FIG. 6.—Antitoxic serum (unconcentrated) from Horse 1351, showing the single line for 3 × crystalline toxin-protein and 3 diffusion lines with the same toxin after treatment with 1 per cent K_3PO_4.

FIG. 7.—Antitoxin (unconcentrated) from Horse 754, showing 2 diffusion lines against 3 × crystalline toxin-protein and 3 lines with phosphate-treated toxin.

FIG. 8.—Special purified antitoxin from Professor Raynaud. Tested against 3 × crystalline toxin-protein and the same toxin after treatment with 1 per cent K_3PO_4.



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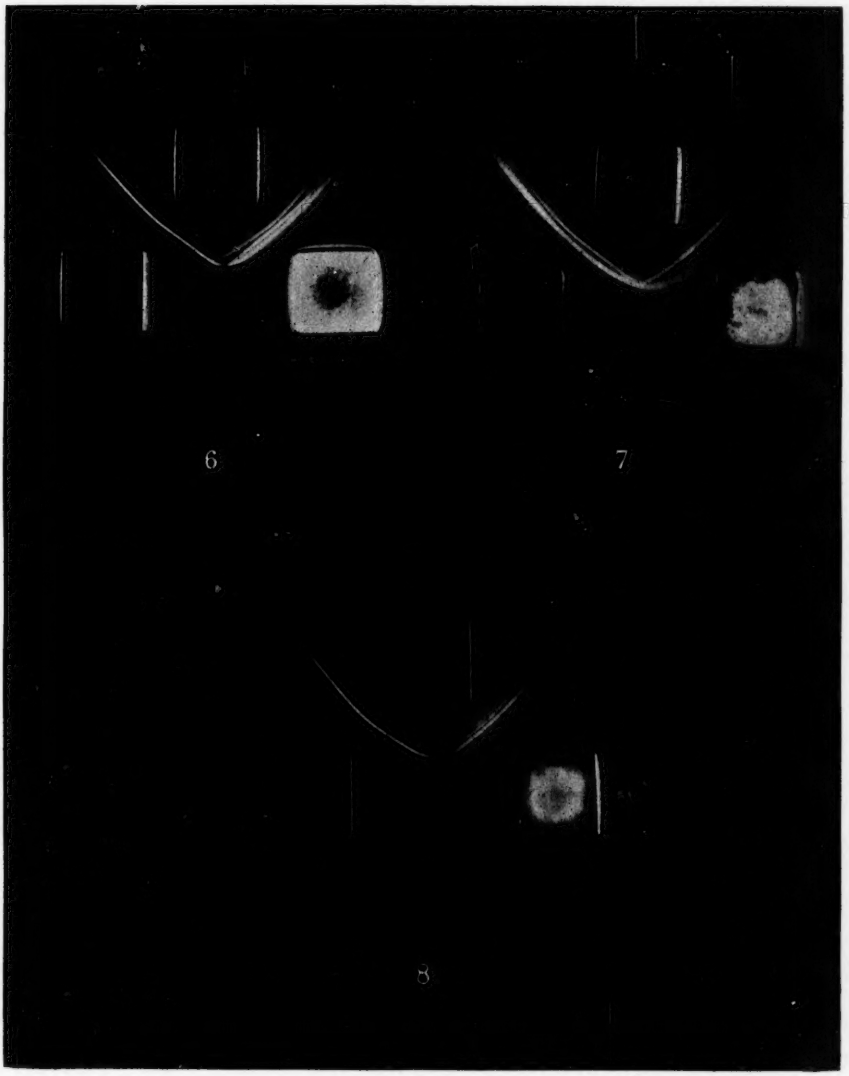

Pope and Stevens.

system there would be two zones, either with excess antibody to antigen or with excess antigen to antibody where precipitation would be incomplete. In the 20 per cent $(NH_4)_2SO_4$ solvent system, the reduced solubility of any complexes formed would lead to the " precipitin " type of curve. The supernatant from the flocculating mixture at the equivalent point was shown to give more precipitate when 20 per cent (w/v) $(NH_4)_2SO_4$ was added, in an amount which accounted for the difference between the two curves.

This point has now been investigated in more detail with the following results :

A mixture was prepared at the equivalence point using crystalline toxin-protein and pepsin refined antitoxin. After heating at 40° for 6 hr. the mixture was kept at 2° for 24 hr. and then centrifuged. The floccule precipitate was washed twice with saline, emulsified in saline and the antibodies liberated by the peptic method of Pope and Healey (1939). From the floccule supernatant, which was slightly opalescent, a second floccule precipitate was obtained by the addition of 20 per cent (w/v) $(NH_4)_2SO_4$. This was removed by centrifuging, washed, emulsified, and the antibodies liberated by peptic treatment. The two preparations, after peptic liberation of the antibodies, were examined by *in vivo*, and *in vitro* methods, and by gel-diffusion tests. The floccule precipitate gave, as expected, a high yield of specific antitoxin. In the gel-diffusion tests it showed a major line with much weaker secondary lines. The antibodies recovered by the ammonium sulphate precipitation of the floccule supernatant showed by *in vivo* tests the presence of only a trace of specific antitoxin ; in the gel-diffusion test 5 lines were observed, but not the major line shown by the floccule-precipitate preparation.

The presence of these antibodies, and the virtual absence of specific antitoxin in the washed precipitate obtained after adding 20 per cent (w/v) $(NH_4)_2SO_4$ to the floccule supernatant, shows that notwithstanding the use of crystalline toxin-protein, antigens differing from the specific toxin must have been present. This result, therefore, supports the views expressed by Pope (1954).

Additional support was also provided by the work of Ambrose and Easty (1954) who studied the gel-diffusion lines by means of an interferometer and found that the major line was complex with at least 4 razor-sharp interference peaks detectable in this band of precipitate, each peak corresponding to a separate antigen-antibody system.

The methods employed to obtain further evidence regarding the antigenic composition of the crystalline toxin-protein fall into 3 groups ; controlled heat denaturation, chemical methods and enzyme treatment.

Controlled Heat Denaturation

If the crystalline toxin-protein consists of several proteins, each with quite distinct antigenic properties, then on heat denaturation these might be destroyed at different rates. This would alter the concentrations present in the original mixture, and the diffusion rates in the gel-diffusion test. This effect was, in fact, observed, and the single line given by the original crystalline material was replaced by a series of lines. Usually 4 distinct lines were obtained ; some samples of crystalline material showed 5 lines. The diffusion tests were carried out using the method of Oakley and Fulthorpe (1953) modified as described by Pope, Stevens, Caspary and Fenton (1951). Two to 5 ml. volumes of toxin, with phosphate buffer (pH 7·5), were heated in boiling water for varying times. Thin walled tubes were used, and the heated toxin was rapidly cooled. Although heat denaturation methods supported the view that the crystalline toxin-protein was heterogeneous

they were difficult to control in different experiments, and have been replaced by more suitable ones.

Chemical Methods

A chance observation led to the first of a series of chemical methods that have been used to demonstrate by gel-diffusion tests the heterogeneity of the crystalline material. Toxin that had been recrystallized using K_2HPO_4 as described by Pope and Stevens (1958) was dialysed and then freeze-dried. It was later shown that the dialysis had been incomplete and that some K_2HPO_4 was present in the material before freeze-drying. When the gel-diffusion test was carried out using the original material, and the redissolved dried-product, it was found that the single line given before drying had been replaced in the case of dried toxin by several diffusion lines.

During the freezing, the removal of water as ice crystals leads to a concentration of the protein and any salts present, conditions not encountered during the previous fractionation, where the highest K_2HPO_4 concentration is about 40 per cent (w/v). For phosphoric acid the value of pK_2 is pH 6·9 and for 99·9 per cent neutralization of the second dissociation constant the value would be pH 9·9. This value is seldom found in solutions of K_2HPO_4, probably due to interaction with atmospheric CO_2, but could be reached under the conditions of freeze-drying due to concentration of K_2HPO_4 and loss of CO_2. With this in mind the action of K_3PO_4 on crystalline toxin-protein was investigated. It was found that as little as 1·0 per cent (w/v) acting for a period of 2 hr. before adjustment of the pH to 8 produced marked changes in the gel-diffusion reaction of crystalline toxins.

In order to shorten the time required for testing these samples the agar plate method of Ouchterlony (1949) at 37° was substituted for the tube method using gelatin at 2–3°. This also had the advantage that the line structure of the original toxin could be compared directly with that of the modified toxin. Fig. 1 shows a typical result obtained in this way. The top reservoir contained pepsin refined antitoxin at 500 u/ml., the lower left reservoir contained thrice crystallized toxin-protein and the lower right reservoir the same toxin after treatment with 1 per cent (w/v) tribasic phosphate for 2 hr. The crystalline toxin-protein produced the characteristic one line while the treated toxin gave 3 lines which all fused with the single line given by the original toxin.

When a higher concentration of K_3PO_4, for example 5 per cent (w/v), was added to the crystalline protein other important changes were found to take place. To a solution of crystalline toxin-protein containing 1000 Lf units/ml. was added 5 per cent (w/v). K_3PO_4; samples were removed at intervals and the pH adjusted to pH 8 by means of phosphoric acid. The flocculation value of these samples was determined in the usual manner. Fig. 2 shows the action of 5 per cent K_3PO_4 on toxin, with time, as determined from the apparent flocculation value and the Lr doses/ml. value. Up to 4 hr. there was little change in flocculation value, but thereafter it changed rapidly to reach a constant at 20 hr. with an apparent Lf value nearly double the initial value. The change in Lr doses/ml. showed an entirely different picture; this fell very rapidly and after 8 hr. it was too low to measure. This effect has been reproducible with all batches of crystalline toxin-protein tested.

When the apparent increase in Lf value has reached its maximum, the product shows only a single diffusion line, has no specific toxicity, and fails to produce

specific antitoxin (*in vivo* tests) after repeated injection into guinea-pigs. At a test level of 50 u/ml. (Kf 50) flocculation occurs in about 1 hr.; it would be accepted by an experienced worker as a normal Ramon flocculation. The optical method of Pope and Stevens (1953*b*) has been used to study the interaction of this, and other modified toxins, with antitoxic sera. These results will be published in detail later. They show quite clearly that treatment with 5 per cent K_3PO_4 results in the survival of one of the antigens which gives a symmetrical flocculation curve peaking in the mixture containing about 50 per cent toxin to antitoxin. The other

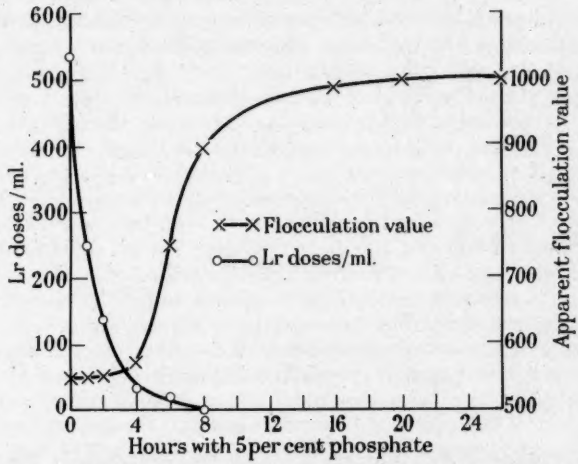

FIG. 2.—The action of 5 per cent K_3PO_4 on crystalline toxin-protein with time, showing loss toxicity and apparent increase in flocculating value.

antigens originally present no longer flocculate although it can be shown that they still combine with their own specific antibodies.

The results described using K_3PO_4 may be duplicated by using tetra-sodium-versenate in a concentration of 1·0 per cent to show multiple diffusion lines or 5 per cent to obtain, with time, the apparent doubling of the Lf value. Both K_3PO_4 and tetra-sodium-versenate at 5 per cent (w/v) produce this apparent doubling of the Lf even though the toxin-protein has been crystallized ten times. A concentration of 0·5 per cent tetra-sodium-versenate also gives separation of the gel-diffusion lines but a longer time is required. The action of 1·0 per cent (w/v) tetra-sodium-versenate on crystalline toxin-protein at 500 u/ml. is shown in Fig. 3 ; the time of action was 2 hr. before adjustment to pH 8. The single line given by the original toxin-protein and the well-separated 3 lines which fuse into this single line are clearly shown.

The high pH values produced by the addition of either K_3PO_4 or tetra-sodium-versenate suggested that similar results might be obtained by cautious addition of NaOH to similar pH values ; this was true for the pH range 11–12 but the results were more variable and NaOH showed no advantage over the 2 reagents previously used.

All these reagents are capable of causing protein denaturation; if distinct antigenic proteins are present, destruction at different rates could alter their original concentrations sufficiently to produce the observed results.

Action of Proteolytic Enzymes on the Toxin-protein

The action of trypsin on crystalline toxin-protein has already been described by Pope (1957). Fig. 4 shows a typical result in which toxin, acted upon by trypsin for 2 days (lower reservoir-left), was compared (lower reservoir-right) with the same material digested for 3 days. This plate illustrates the individual nature of each diffusion line and the specific nature of each antibody involved.

Similar results were obtained when the crystalline toxin-protein was attacked with pepsin. At the pH optimum for this enzyme (pH 1–2), all immunological activity is rapidly destroyed. However, at less acid pH values pepsin produces results similar to those produced by trypsin as shown by the following experiment. To 100 ml. of crystalline toxin-protein at 1000 u/ml. 5 mg. crystalline pepsin was added and the pH adjusted at once to 4·7. A heavy precipitate formed and more acid was added cautiously until this had nearly all dissolved. After 6 hr. digestion at 16° a sample was neutralized to pH 8; it showed two flocculation zones with apparent Lf values of 400 and 700 units and gave the gel-diffusion test shown in Fig. 5. Both lower reservoirs contained the digested toxin and it is seen that the 3 diffusion lines were quite independent of each other. Based on considerable work with pepsin the pH optimum for this digestion was found to be 3·7.

Papain and crystalline carboxypeptidase, failed to produce changes of the kind shown by trypsin and pepsin. Crystalline chymotrypsin showed only a weak activity which might have been due to slight contamination with trypsin.

Variation of the Antitoxin Used for the Gel-diffusion Tests

The antitoxin used initially in these tests was routine pepsin-refined antitoxin prepared from a bulk blending of the serum of different horses. Several such batches of antitoxin showed similar results, but it was considered that the blending of sera would give average values for different specific antibodies, and so obscure any variation in their production by the hyperimmunized horse. Sera obtained from individual horses under immunization were therefore examined by the gel-diffusion method against crystalline toxin-protein and the same toxin treated with 1 per cent (w/v) K_3PO_4 for 2 hr. From these tests it was at once clear that individual horses had responded differently in their production of antibodies to the different antigens. Against the same " phosphate-toxin " some sera gave 2, others 3 and a few gave 4 diffusion lines. In some cases the lines were well separated, but in others were close together. Of far greater interest was the finding that several of these horse sera gave 2 lines with the original crystalline-toxin-protein used as the control, instead of the one previously found when pepsin refined antitoxin was employed. The results obtained from a series of 37 samples of serum from individual horses undergoing hyperimmunization is given in the Table. Their antitoxic values ranged from 180 to 1850 u/ml. but no clear connection between this value and the diffusion pattern could be found. Fig. 6 shows antitoxic serum (500 u/ml.) against thrice crystallized toxin-protein (single line) and the phosphate-treated toxin (3 lines). An example of the production of 2 lines with the control crystalline toxin-protein is shown in Fig. 7.

TABLE.—*The Gel-diffusion Lines Produced by Sera from 37 Individual Horses Against Crystalline Toxin-protein and Phosphate-treated Crystalline Toxin-protein*

Crystalline toxin	Phosphate-treated toxin*
32 horses—1 line	12 horses—2 lines
	19 ,, 3 lines
5 ,, 2 lines	6 ,, 4 lines

* 1 per cent K_3PO_4 for 2 hr.—then neutralized.

Another striking example was provided through the examination of a special sample of antitoxin provided by Professor Raynaud. After isolation of the antitoxin in the β_2 globulin fraction he precipitated it with purified toxin (3000 Lf/mg. N); the washed floccules were submitted to peptic action and the antibodies recovered. By the usual diffusion tests this appeared to be a pure single-line antitoxin. When tested by us against 3 times crystallized toxin, and the phosphate-toxin prepared from it, the result shown in Fig. 8 was obtained; at least 3 distinct antibodies are present in this antitoxic material.

DISCUSSION

The work reported by Pope (1954) showing the interaction between toxin and antitoxin in 2 different solvents has been extended to show that the additional precipitate, produced in the supernatant from the equivalent (neutral) mixture on the addition of 20 per cent (w/v) ammonium sulphate, contained too little specific antitoxin to be detected after pepsin treatment; other antibodies giving gel-diffusion lines were present. If the difference between the "flocculation" and "precipitin" curves reported by Pope (1954) were merely due to incomplete precipitation of the floccules, it would not be expected that the additional precipitate, produced by $(NH_4)_2SO_4$, would be free of specific antitoxin, and yet contain other easily demonstrated antibodies. This finding supports the view that the crystalline toxin-protein contains a series of distinct antigens all reacting with their own antibodies and that they are not all equivalent to each other at the same point. Each antigen requires its own volume of the antitoxic serum for optimal precipitation.

By a number of methods, described in this paper, the crystalline toxin-protein may be altered so that it shows a number of diffusion lines in the gel-diffusion test. It is important to realize that all the antitoxic sera used were produced by the injection of untreated toxin or toxoid and not by means of toxin submitted to any of the methods described in this paper. The presence of distinct, and specific antibodies is, therefore, a normal feature in antitoxic sera and the results presented for individual horse sera indicate tht they may be produced in varying amounts.

The observations made by Ambrose and Easty (1954) indicate that the major diffusion line is built up of at least 4 distinct antigen-antibody systems and that these antigens have independent existence in the original toxin-protein. This is also supported by the work of Poulik (personal communication) who, by means of electrophoresis in a starch-gel system, has separated the components in crystalline toxin-protein. It is, therefore, not considered that any of the methods described in this paper results in the breakdown of a large molecule, to give rise to a series of antigenic artefacts. A change in the relative concentration of the

different antigens is considered to be the reason why they may be detected by the gel-diffusion methods.

Pope and Stevens (1958) have shown that during the purification of toxin the specific toxin is readily adsorbed on to other proteins, and have described methods for avoiding this to a large degree. However, from the results given here, and other (unpublished) work there is still reason to believe that the specific toxin is associated with other proteins all of which have very similar physical-chemical characteristics.

The production by *C. diphtheriae* (Park Williams 8 Strain) of a series of proteins so closely associated with the specific toxin is remarkable, but not explainable, at the present time. There seems to be little doubt that toxins produced in other laboratories contain a similar series of antigenic proteins associated with the specific toxin since antitoxic sera from other sources (America and France) contain the antibodies to them. The antitoxin No. 5353 A.D. used by Pappenheimer and Yoneda (1957) and by Pappenheimer in his earlier work, was shown by Pope (1957) to give 2 flocculation zones in the Ramon test, and 3 diffusion lines, when tested against crystalline toxin-protein partially digested with trypsin. Of the 3 diffusion lines, two were of about equal intensity and the third rather weak. It is known that this antitoxin was rather exceptional; the horses from which it was obtained were only immunized for a short time and a maximal antibody response was probably not obtained. The examination of sera from 37 horses immunized to diphtheria toxin shows that the different antibodies are not produced in constant amounts relative to each other. By selection it is possible to obtain sera showing the maximum separation of the diffusion lines when tested against crystalline toxin-protein treated as described here. In 5 sera of this group the relative amounts of these antibodies was such that the crystalline toxin-protein showed more than one diffusion line.

The observations reported by Lapresle (1955) who studied the action of an enzyme from rabbit spleen on human serum albumin are similar to those reported here for the crystalline toxin-protein. He found that the sera of rabbits immunized with human serum albumin contained 3 antibodies which reacted specifically with human serum albumin that had been acted upon by the spleen enzyme. The 3 antigens obtained by enzyme action had slightly different electrophoretic mobilities. Lapresle considered that the antigenic pattern of human serum albumin comprises three different specific groupings which are separated by enzyme action at pH 3 to yield degradation products with specific antigenic properties. This interpretation differs from that held for the composition of the crystalline toxin-protein where it is considered that all the antigens exist as entities and not as part of a complex molecule.

Attempts have been made to separate the antigens by chemical and physical methods. The specific toxin has, naturally, received most attention, but its labile nature has made experimental work very difficult. More progress has been made with the isolation of one of the non-toxic antigens and this will be reported later.

SUMMARY

Through the use of methods which cause partial denaturation of the proteins present in crystalline diphtheria toxin-protein it has been possible to demonstrate, by gel-diffusion methods, its antigenic complexity. At least 3 and often 4 distinct antigens are detectable in this way.

Under certain conditions the apparent Lf value of the crystalline toxin-protein may be doubled ; the reasons for this apparent increase are given.

A study of sera from horses immunized to diphtheria showed that they contained the specific antibodies to the different antigens. These antibodies are produced in different relative amounts in different horses.

We would thank Professor M. Raynaud for the gift of the special antitoxin derived from the β_2 globulin fraction, and Mr. E. A. Jones for the photographs of the diffusion plates.

REFERENCES

AMBROSE, E. T. AND EASTY, G. C.—(1954) *Disc. Faraday Soc.*, **18**, 364.
LAPRESLE, C.—(1955) *Ann. Inst. Pasteur*, **89**, 654.
OAKLEY, C. L. AND FULTHORPE, A. J.—(1953) *J. Path. Bact.*, **65**, 49.
OUCHTERLONY, O.—(1949) *Ark. Kemi. Min. Geol.*, *B***26**, 1.
PAPPENHEIMER, A. M. AND YONEDA, M.—(1957) *Brit. J. exp. Path.*, **38**, 194.
POPE, C. G.—(1954) *Disc. Farday Soc.*, **18**, 323.—(1957) *Brit. J. exp. Path.*, **38**, 207.
Idem AND HEALEY, M.—(1939) *Brit. J. exp. Path.*, **20**, 213.
Iidem AND STEVENS, M. F.—(1953*a*) *Lancet*, ii, 1190.—(1953*b*) *Brit. J. exp. Path.*, **34**, 56.
Iidem, CASPARY, E. A. AND FENTON, E. L.—(1951) *Brit. J. exp. Path.*, **32**, 246.

AN EXPERIMENTAL DEMYELINATIVE DISEASE IN THE SYRIAN HAMSTER*

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IN 1933 an experimental disease, acute allergic disseminated encephalomyelitis, was successfully produced in monkeys by the repeated injection of brain tissue suspensions. Histopathologically and clinically the condition resembled in certain respects the acute demyelinative encephalitides of man (Rivers and Schwentcker, 1935; Rivers, Sprunt and Berry, 1933). This experimental disease has also been produced in other laboratory animals, namely, rabbits (Morrison, 1947; Kopeloff and Kopeloff, 1947), guinea-pigs (Freund, Stern and Pissani, 1947; Jervis and Koprowski, 1948; Alvord, 1949), dogs (Thomas, Paterson and Smithwick, 1950; Jervis, Burkhart and Koprowski, 1949; Koprowski, Jervis, James, Burkhart and Poppensiek, 1950) and white mice (Olitsky and Yager, 1949). Different species may show slightly different histopathological pictures, both as to the topographical distribution of the lesions and, more particularly in their readiness to respond by demyelination (Hurst, 1952). A species variability indicates the existence of endogenous factors in the development of the experimental disease, which seems to be of importance in human multiple sclerosis. Prevailing theories concerning the pathogenesis of the latter condition also emphasize constitutional or endogenous factors (Olitsky Casals and Tal, 1950; Olitsky and Tal, 1951). In the light of these considerations and in an attempt to expand our knowledge of species differences in susceptibility, we have studied the experimental disease in the Syrian hamster.

MATERIALS AND METHODS

The preparation of the injection materials and also the experimental procedure adopted closely resembled those already described (Olitsky an Yager, 1949; Olitsky and Tal, 1952). A brief outline, however, will be provided for clarification.

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EXPLANATION OF PLATES

FIG. 1.—Perivascular monocytic infiltration of pial blood vessels penetrating into brain substance. × 125. Hæmatoxylin and Eosin.

FIG. 2.—Granulomatous inflammation of cornu ammonis. × 520. Hæmatoxylin and Eosin.

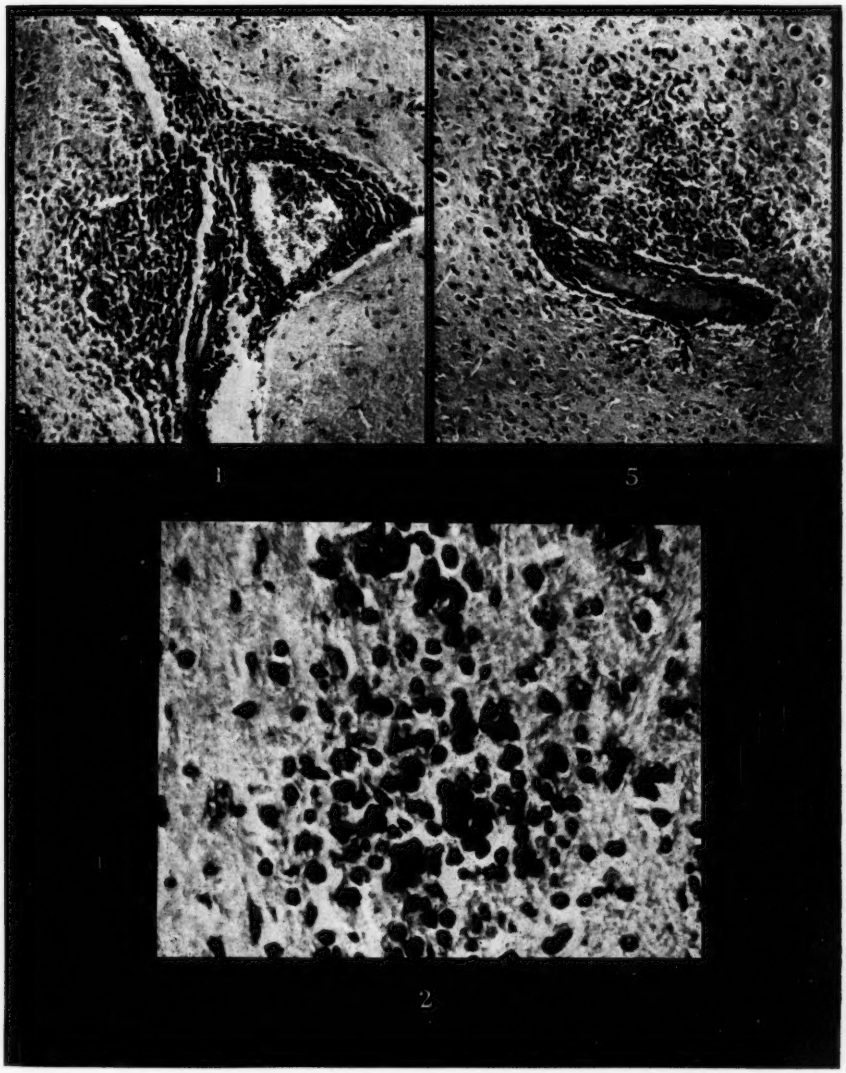
FIG. 3.—Demyelinative plaque in long tract of pons. × 150; Weil-Weigert's method for myelin.

FIG. 3A.—The ruled area shows the region photographed in Fig. 3.

FIG. 4.—Demyelinative plaque in white matter of cerebellum. × 150. Weil-Weigert's method for myelin.

FIG. 4A.—The ruled area shows the region photographed in Fig. 4.

FIG. 5.—Granulomatous inflammation, peri- and juxtavascular. × 125. Hæmatoxylin and Eosin.



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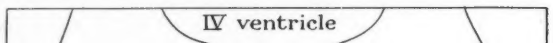
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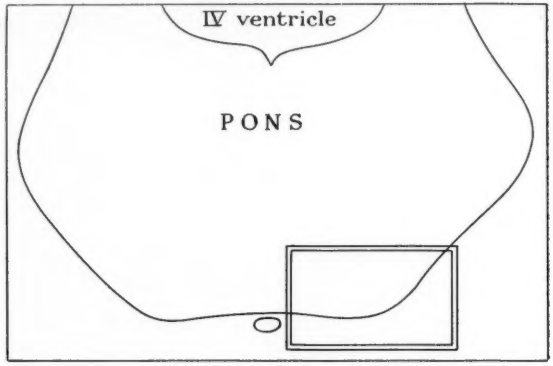

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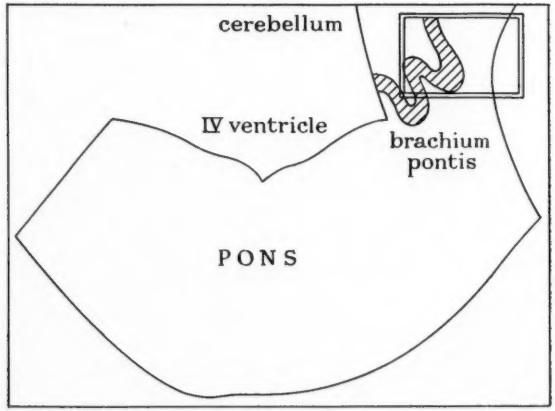

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Preparation of adjuvant.—The adjuvant used was the Freund-type variant (Freund and McDermott, 1942) consisting of 50 ml. of mineral oil (Byol F), 50 ml. of physiological saline, 250 mg. of autoclaved tubercle bacilli, type H37RV, and 20 mg. of merthiolate mixed in a Waring blender for 4 min. and thereafter kept at 4° during the period of the experiment. All manipulations were done under aseptic conditions.

Preparation of proteolipid-adjuvant mixture.—The proteolipid A.B. fraction (Folch and Lees, 1951) was prepared from fresh whole brain (homologous or heterologous). The yield from 10 g. of fresh whole brain (which was equivalent, as a rule, to 100 mg. of proteolipid, dry weight) suspended in saline, was incorporated into 100 ml. of adjuvant.

Preparation of whole-brain-adjuvant mixture.—10 g. of fresh whole brain (homologous or heterologous) were incorporated into 100 ml. of adjuvant by mixing in a Waring blender for 4 min.

The animals used were Syrian hamsters of a local stock (Adler, 1948). The morbidity and mortality rates in this stock under ordinary laboratory conditions are very low and these animals are also extremely resistant to epidemic outbreaks of an infectious nature. Young adults of both sexes were used; they were about 3 months old and each weighed about 80 g.

Experimental group

This consisted of 60 animals; of these, 40 are referred to as "early stage animals", the survivors being killed 40 to 70 days after the beginning of the experiment; 20 are referred to as "later stage animals", the survivors being killed 140 days after the beginning of the experiment. In each group some animals died earlier than the stated periods; in the case of the "early stage animals" these were included in the present study (see Table I); in the case

TABLE I.—*Details of Injection Experiments*

Sub-group	Number of animals injected	Material injected	Method of injection	Number of animals which died	Number of survivors	Number of days between first injection and killing of survivors
			"Early Stage" Animals			
A	16	Homologous proteolipid-adjuvant mixture	1 ml. weekly for 6 weeks. Subcutaneous	2 (5)* 4 (12) 2 (14)	8	40
B	7	Homologous-whole-brain-adjuvant mixture	A single 1 ml. injection. Intramuscular	0	7	46–70
C	12	Whole mouse brain-adjuvant mixture	1 ml. weekly for 6 weeks. Subcutaneous	1 (14) 3 (18) 1 (32)	7	60
D	5	Homologous whole-brain-adjuvant mixture	1 ml. at beginning of experiment and 1 ml. 45 days later. Subcutaneous	0	5	70
			"Later Stage" Animals			
A	13	Homologous proteolipid-adjuvant mixture	1 ml. weekly for 6 weeks. Subcutaneous	—	10	140
C	12	Whole mouse brain-adjuvant mixture	1 ml. weekly for 6 weeks. Subcutaneous	—	8	140

"Later stage" animals dying earlier than 140 days after the first injection were discarded; thus, no particulars of these animals are given in the above table.

* The figures in parentheses are number of days between first injection and day of death.

of the " later stage animals " they were discarded. Both early and later stage animals were divided into subgroups according to the method of injection and the material used; various procedures were adopted to try and determine which was the most effective. Details are given in Table I.

Control groups

A group of 50 hamsters were each given a weekly subcutaneous injection of adjuvant only for 6 weeks. Early stage controls were killed 70 days after the first injection and later stage controls 140 days after the first injection.

A further group of 10 hamsters were treated in the same way with physiological saline only and killed 140 days after the first injection.

In order to rule out the possibility of viral infection the following control experiment was conducted. Whole hamster brain suspension was prepared by blending 20 g. of whole brain from 3-month-old animals in 100 ml. of physiological saline containing 10 per cent inactivated (56° for 30 min.) rabbit serum. To this were added 10,000 units of penicillin and 10 mg. streptomycin. This suspension was then injected into 10 Syrian hamsters (3 months old), 15 baby mice (21 days old) and 10 8-day chick embryos. The hamsters were each injected intracerebrally with 0·05 ml., the mice intracerebrally with 0·03 ml., and into the yolk sac of each embryonated egg was injected 0·5 ml. The hamsters and mice were observed for 2 months after injection. Examinations of brain sections from experimental as well as control animals for the presence of *encephalitozoon cuniculi* were negative.

Histological Technique

The brain and spinal cord were fixed in 10 per cent formalin (3 to 10 days). Thereafter, coronal sections were made through the brain and transverse sections through the spinal cord at various levels. Paraffin sections were stained with haematoxylin and eosin, Gram's stain and for myelin by the Weil-Weigert, Spielmeyer, Loyez and Luxol-fast blue (Everson-Pearse, 1955) methods.

TABLE II.—*Clinical and Histopathological Observations*

Subgroup as in Table I	Number of animals injected	Number of animals with clinical CNS signs		Number of animals without clinical CNS signs	Number of animals with CNS histopathological lesions	
		Paralytic	Non-paralytic		Inflammatory	Demyelinative
		" Early Stage " Experimental Animals				
A	16	7	15	0	16	0
B	7	3	6	0	7	0
C	12	5	8	6	12	0
D	5	0	0	5	5	0
		" Later Stage " Experimental Animals				
A	10	7	6	0	10	10
C	10	6	8	0	10	10
		" Early Stage " Controls. Injected with Adjuvant Only.				
—	30	0	0	30	17	0
		' Later Stage " Controls. Injected with Adjuvant Only.				
—	10	0	0	10	7	0
		Controls Injected with Saline Only.				
—	10	0	0	10	0	0

RESULTS

Clinical Observations

Neurological signs developed in some of the " early stage " and in all the " later stage " experimental animals (Table II). The signs were either paralytic or non-paralytic or both. The former included weakness, paresis and paralysis of limbs ; sometimes the urinary bladder and the rectum were also affected. Non-paralytic signs consisted of tremor, ataxia and circling. A number of animals developed constitutional reactions such as loss of weight, ruffled fur, hunched posture, respiratory difficulty, and copious non-purulent secretion from eyes and nose.

None of the control animals showed any neurological or constitutional signs and the chick embryos hatched normally.

Histopathological Findings

" Early stage " experimental animals

Lesions were found in all the animals. In all instances the brain as a whole was involved but there was great variability in the intensity and extent to which individual animals were affected. The same histopathological picture was found in the various subgroups.

The pia-arachnoid membranes were thin and in and beneath them small foci of mononuclear cells were frequently present. In some areas these cells were diffusely scattered ; in others they followed and surrounded the small cortical vessels (Fig. 1). In the brain tissue were scattered many granulomatous lesions of varying size, consisting of lymphocytes, histiocytes, plasma cells and occasional granulocytes (Fig. 2). Some of these infiltrations were in the form of perivascular cuffs which penetrated through the pial-glial membrane into the brain substance. In other instances these foci were in juxtaposition to blood vessels. There was degeneration and shrinking with satellitosis of some neurones. No areas of demyelination could be detected.

In the spinal cord similar inflammatory lesions were found in the anterior horns of the grey matter in some animals.

" Later stage " experimental animals

The histopathological pictures in the two subgroups were similar. Multiple inflammatory lesions were found in all the animals identical with those observed in the " early stage " animals ; these were usually found in juxtaposition to small blood vessels or around capillaries. The lesions were granulomatous in that they consisted of a variety of so-called inflammatory cells such as granulocytes, lymphocytes, many plasmacytes of a more or less mature type and histiocytes. In contrast to the " early stage " animals, however, myelin stains showed in all the animals demyelination in a narrow rim around lesions which happened to be within the white matter. The myelin stains also showed small demyelinative plaques which were best revealed where tracts were cut longitudinally. The patches of demyelination usually affected only part of the length and breadth of the tract (Fig. 3 and 4). Again, great individual variability was observed in the intensity of the reactions, both inflammatory and demyelinative.

No parallelism was found either in the " early " or " later stage " animals between the intensity and distribution of the histopathological findings and the clinical manifestations.

Controls

In 17 of the 30 animals injected with adjuvant alone, there were found inflammatory lesions in the CNS, identical morphologically and topographically with those of the experimental animals (Fig. 5). No demyelinative lesions were found.

The animals injected with saline alone showed no histopathological changes whatever, nor did those injected intracerebrally with whole hamster brain suspension.

DISCUSSION

As shown by earlier authors, experimental encephalitis can be produced in different species of animals by the injection of homologous or heterologous brain or brain proteolipid, mixed with the Freund-type adjuvant. The results of the present investigation indicate that the Syrian hamster is also susceptible to the experimental disease ; one animal showed inflammatory lesions as early as 5 days after a single injection (Table I).

It is of interest that an inflammatory process was the only pathological feature in the " early stage " experiment, i.e. up to 70 days after the first injection. At " later stages ", however, i.e. 140 days after the first injection demyelinization was found in addition to the inflammatory lesions ; the latter were still present 98 days after the last injection.

The great variability of individual susceptibility may be due to the fact that the animals used were not inbred.

It was shown by Rivers (Rivers and Schwentcher, 1935 ; Rivers *et al.*, 1933) that in experimental animals repeated injections of brain tissue alone can elicit the condition described as allergic encephalomyelitis ; however, in such animals the CNS reaction to the injections is slow and for this reason later authors (Morgan, 1947 ; Kabat, Wolf and Bezer, 1947) added Freund's adjuvant to the brain tissue as a means of enhancing the reaction of the CNS.

In the present experiments the effect of adjuvant when injected alone was also investigated. It was found that single or repeated injections of adjuvant alone produced in the brains of most of the animals inflammatory changes indistinguishable morphologically from those produced by injections of whole brain or brain proteolipid-adjuvant mixture. At no time, however, was demyelinization noted, although the inflammatory foci persisted. These results suggest that the specific lesions in experimental acute allergic encephalomyelitis in the hamster are the demyelinative and not the inflammatory foci. " Experimental demyelinative disease " might therefore be a more appropriate name for the condition than " experimental encephalomyelitis ".

As to the mechanism of the demyelinization : the distribution of the demyelinative lesions, their appearance as patches within tracts and their absence in animals injected with adjuvant alone would suggest their production by a specific active process resulting from the administration of brain substance rather than by passive demyelinization of axis cylinders injured by the inflammatory lesions.

A problem yet to be resolved is the presence of clinical signs of CNS involvement in the " early stage " experimental animals (Table II) but their absence in controls injected with adjuvant only, even though the histopathological lesions observed were identical in these two groups of animals. It is possible that in the " early stage " animals demyelinative changes are already present which, however, were not detected by the histological methods used. If this suggestion be correct and the experimental disease should be shown to be primarily demyelinative even though inflammatory lesions be present, then it would appear to bear a resemblance to spontaneous primary demyelinative processes in humans.

The variation in the susceptibility of difierent animal species to the experimental disease has already been mentioned. It may be that this variation is similar to that of different human groups to primary demyelinative processes. It is possible that this variation in human susceptibility is a species variant of a reaction to factors which basically are similar to those producing the experimental disease, *i.e.*, that the reaction in humans is the result of sensitization to the patient's own brain tissue.

SUMMARY

An experimental demyelinative disease has been produced in the Syrian hamster by the injection of whole-brain homogenate-adjuvant mixture or proteolipid-adjuvant mixture.

Animals killed 140 days after the first injection showed demyelinative changes in addition to inflammatory foci in the CNS. In animals dying or being killed up to 70 days after the first injection inflammatory foci only were observed.

Injections of adjuvant alone produced inflammatory changes identical with those produced by whole brain or proteolipid injections, but neither demyelination nor clinical signs of CNS involvement were observed.

It is suggested that the specific lesions in the condition produced by whole brain or proteolipid are demyelinative and not inflammatory.

The similarity between the histopathological pictures in the experimentally produced disease and human primary demyelinative disease is noted and the implications discussed.

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REFERENCES

ADLER, S.—(1948) *Nature*, **162**, 256.
ALVORD, E. C.—(1949) *J. Immunol.*, **61**, 355.
EVERSON-PEARSE, A. G.—(1955) *J. Path. Bact.*, **70**, 554.
FOLCH, J. AND LEES, M.—(1951) *J. biol. Chem.*, **191**, 807.
FREUND, J. AND MCDERMETT, K.—(1942) *Proc. Soc. exp. Biol., N.Y.*, **49**, 548.
Idem, STERN, F. R. AND PISSANI, T. M.—(1947) *J. Immunol.*, **57**, 179.
HURST, E. W.—(1952) *Amer. J. Med.*, **12**, 547.
JERVIS, G. A., BURKHART, R. L. AND KOPROWSKI, H.—(1949) *Amer. J. Hyg.*, **50**, 14.
Idem AND KOPROWSKI, H.—(1948) *J. Neuropath.*, **7**, 309.

KABAT, E. A., WOOLF, A. AND BEZER, A. E.—(1947) *J. exp. Med.*, **85**, 117.
KOPELOFF, L. M. AND KOPELOFF, N.—(1947) *J. Immunol.*, **57**, 229.
KOPROWSKI, H., JERVIS, G. A., JAMES, T. R., BURKHART, R. L. AND POPPENSIEK, C.C. —(1950) *Amer. J. Hyg.*, **51**, 63.
MORGAN, J. M.—(1947) *J. exp. Med.*, **85**, 131.
MORRISON, L. R.—(1947) *Arch. Neurol. & Psychiat.*, **58**, 391.
OLITSKY, P. K., CASALS, J. AND TAL, C.—(1950) *Proc. Soc. exp. Biol., N.Y.*, **75**, 276.
Idem AND TAL, C.—(1951) *Ibid.*, **78**, 607.—(1952) *Ibid.*, **79**, 50.
Idem AND YAGER, R. H.—(1949) *J. exp. Med.*, **90**, 213.
RIVERS, T. M. AND SCHWENTCKER, F. F.—(1935) *Ibid.*, **61**, 689.
Idem, SPRUNT, D. H. AND BERRY, G. P.—(1933) *Ibid.*, **58**, 39.
THOMAS, L., PATERSON, P. Y. AND SMITHWICK, B.—(1950) *Ibid.*, **92**, 133.

STAPHYLOCOAGULASE ACTIVITY *IN VIVO*

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THE ability of certain staphylococci to clot plasma was demonstrated as early as 1908 (Much), but it was many years before it became recognised that coagulase production was peculiar to *Staph. aureus* and therefore could be used as a criterion of pathogenicity (Cruickshank, 1937). At the present time the *in vitro* production of coagulase is the commonest and most important test used to differentiate pathogenic and non-pathogenic staphylococci.

Menkin and Walston (1935) made the first attempt to determine whether coagulase production was merely an *in vitro* character or whether it also played a part in the mechanism of disease. Unlike streptococcal lesions which tend to spread, those due to *Staph. aureus* are characteristically localised. Menkin and Walston suggested that this localisation might be due to a fibrin barrier resulting from coagulase action. They failed, however, to show *in vivo* clotting by coagulase and concluded that although the barrier existed, it was due to toxic action on the surrounding tissues. Subsequently Fisher (1936) also failed to find evidence of coagulase action *in vivo*.

Strong presumptive evidence for the *in vivo* action of coagulase came from the work of Wilson Smith and his colleagues (Hale and Smith, 1945 ; Smith, Hale and Smith, 1947), who found that plasma clotted by coagulase inhibits phagocytosis of staphylococci. They also demonstrated that clotting of plasma *in vivo* might be a pre-requisite for the establishment of staphylococcal infection. In their experiments strains of staphylococci capable of clotting human but not guinea-pig plasma did not cause more than a transient lesion in guinea-pigs, but when the organisms were injected at a site into which human plasma had been previously injected, multiple abscesses developed. Lominski and Roberts (1946) showed that the serum of many healthy people contained a substance which prevented coagulase clotting *in vitro*: this inhibitory substance was absent from patients who had recently suffered from a major staphylococcal infection such as acute osteomyelitis. Sera with high anti-coagulase titres protected rabbits against experimental infection (Lominski, 1949). Coagulase inhibition by human serum was subsequently demonstrated by a number of other workers (Kaplan and Spink, 1948 ; Rammelkamp, Badger, Dingle, Feller and Hodges, 1949 ; Tager and Hales, 1948*a*). Finally it was found that animals actively immunised against coagulase were also protected from infection (Boake, 1956).

In the face of all this evidence there can be little doubt that coagulase plays a part in staphylococcal infection, but there is no direct evidence that it does so by exerting its clotting action. The nearest approach to this is possibly the work

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of Tager (1954) who found that after the injection of massive doses of coagulase rabbits died with lowered fibrinogen levels in the blood.

We believe that the present work briefly reported earlier (Smith and Johnstone, 1956) provides direct evidence that coagulase may produce clotting *in vivo*. This clotting action was judged both by the estimation of rabbit blood fibrinogen levels before and after intravenous coagulase injections, and by the histological demonstration of intravascular fibrin deposits in rabbits after these injections.

MATERIALS AND METHODS

Coagulase

Three preparations of coagulase were used. Coagulases 1 and 2 were dried, purified preparations obtained from the well-known coagulase-producing strain Newman: they contained no alpha toxin, being non-haemolytic for rabbit red cells and producing no dermonecrosis in rabbits. Both were very potent: coagulase 1 at a dilution of 1 in 500,000 clotted human plasma diluted 1 in 10 in 1 hr.; coagulase 2 was approximately four times more active. Although highly purified these preparations were not pure and contained only approximately 5 per cent of coagulase (Duthie, personal communication).

Coagulase 3 was a crude preparation from our own high coagulase-producing strain 5R. It was the supernatant of 4-day broth cultures and clotted human plasma at a dilution of 1 in 2000 in 1 hr. This preparation was used for *in vitro* titrations only.

Each of these coagulase preparations clotted not only human but also mouse and rabbit plasma. As measured by clotting times the potency against rabbit and human plasma was the same, but against mouse plasma it was about 16 times lower.

Coagulase injection

Each dose of purified coagulase was dissolved in 5 ml. of saline and slowly injected (10–20 min.) into the ear vein of rabbits. In mice the coagulase, dissolved in 0·5 ml. of saline, was injected into the tail vein.

Animals

Chinchilla rabbits weighing 2–4 kg. and the Porton Swiss White strain of mice were used.

Rabbits immunized against coagulase

Animals were immunised with a coagulase similar to coagulase 3; it was the supernatant of 4-day fluid cultures of strain 5R. In addition to coagulase it also contained gamma lysin. Initially 0·2 ml. of this preparation was injected intracutaneously, followed at weekly intervals by 2 subcutaneous, and 3 intravenous injections, each of about 0·75 ml. The animals were bled 10 days after the last injection and the serum examined for its anti-coagulase titre.

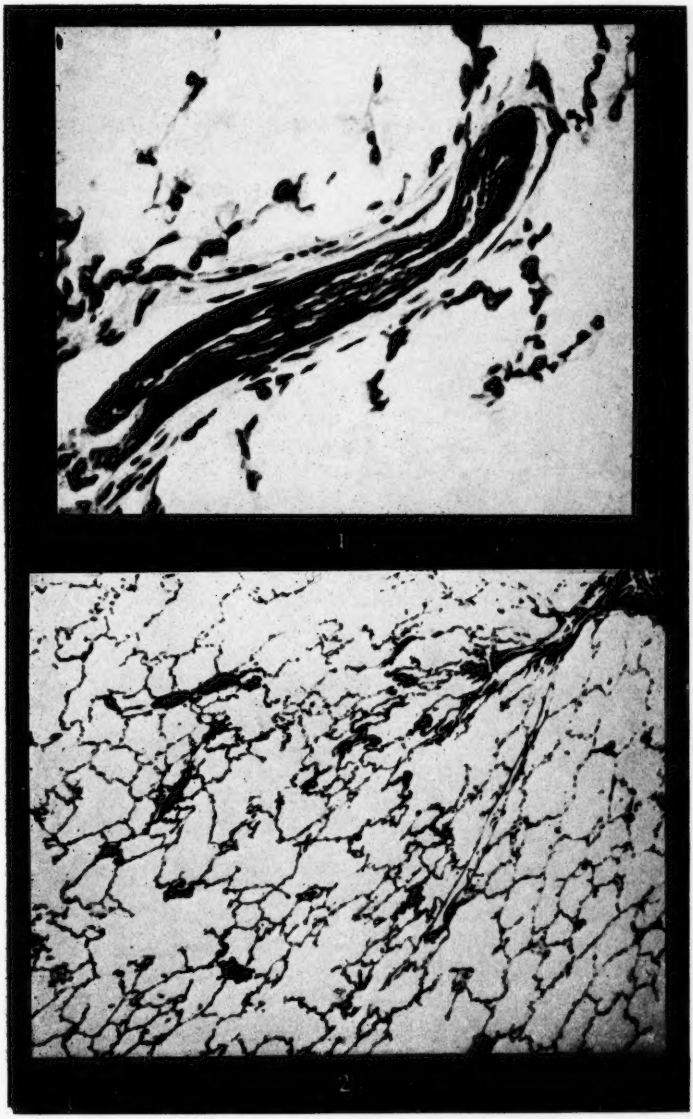
Titration of coagulase inhibition of rabbit sera

The titration was that devised by Lominski and Roberts (1946). Constant amounts of coagulase together with doubling dilutions of the serum being tested were incubated at 37° for 90 min.; thereafter human plasma was added to a final dilution of 1 in 10 and the highest inhibiting dilution of serum was recorded. Readings were taken at the time the control clotted.

EXPLANATION OF PLATE

FIG. 1.—After intravenous coagulase injection the resulting fibrin deposits are formed of multiple strands, ranged parallel to one another and to the axis of the occluded vessel. Gram. × 480.

FIG. 2.—This representative lung field from a rabbit killed by intravenous injection of coagulase shows occlusion of the majority of capillaries and vessels by dark fibrin deposits. Masson's trichrome. × 100.



Smith and Johnstone.

Estimation of blood proteins

Fibrinogen was estimated by the biuret method described by Barry, Feeney and Geoghegan (1955): heparin was found to be preferable to oxalate as anticoagulant. The method of Gornall, Bardawill and David (1949) was used for the estimation of albumin and globulin.

Histological demonstration of fibrin

Rabbits died within 30 min. of the intravenous injection of large doses of coagulase (*i.e.* 10 mg. of coagulase 1 or 3 mg. of coagulase 2), or were killed at 30 min. after the injection of smaller doses. After preliminary fixation in 10 per cent formal saline the organs were fixed for 24 hr. in corrosive formol. Paraffin sections were prepared and stained by haemalum and eosin, Gram's method, Masson's trichrome, Picro-Mallory, eosin phloxine and tartrazine and Liebs' phosphotungstic acid haematoxylin.

RESULTS

The intravenous injection of coagulase in rabbits resulted in a marked and very rapid fall in fibrinogen with widespread intravascular clotting affecting particularly the lungs.

Effect of coagulase on blood fibrinogen levels

Blood fibrinogen levels before and after the intravenous injection of coagulase 1 are shown in Table I. The slow administration of small amounts had little general effect on the animals, but, if given quickly, they became very agitated and developed leg weaknesses for a short period. After the administration of a large amount the animals rapidly became extremely excited before collapsing with violent spasmodic leg movements and gasping respirations preceding death. In all animals the puncture wounds in the ears bled profusely and bleeding was difficult to control.

TABLE I.—*Effect of Coagulase I on Rabbit Blood Fibrinogen Levels when Injected Intravenously*

Rabbit No.	Coagulase 1 (mg.)	Fibrinogen level (mg./100 ml.) Zero	30 min.	1 day	2 days	3 days	4 days	6 days	Remarks
F2	2	270	95	400	460	360	370	265	Mild reaction.
F6*	4·5	270 (7200)	40 (6300)	320 (5800)	500 —	410 (5800)	435 (5800)	330 (6000)	No apparent effect.
F4	5	300	60	410	—	550	—	—	,, ,,
D30	5	270	110	410	670	525	—	290	Severe reaction.
D31	10	185	25	—	—	—	—	—	Death (½ hr.).
W6	10	480	18	—	—	—	—	—	,, ,,
E54	6 autoclaved	330	305	—	—	—	—	—	No effect: autoclaved coagulase 1.

* Figures in brackets record total protein values in mg./100 ml.

After injection the blood fibrinogen level fell very rapidly, reaching its lowest point at about 30 min., then it quickly rose until by 48 hr. it had reached approximately twice the original value. The level slowly returned to normal in 6 to 8 days (Table I).

Blood samples were also examined electrophoretically and by this means the fibrinogen values obtained chemically were confirmed. It was also shown that fibrinogen alone was being estimated. Further, electrophoresis of the plasma after chemical removal of fibrinogen, confirmed that the fibrinogen was being estimated completely.

A sample of coagulase 1 inactivated by autoclaving for 60 min. was found to have no *in vitro* clotting power. Intravenous injection of 6 mg. had no apparent effect on the rabbit and its fibrinogen level was not altered significantly (Table I).

Effect of coagulase on plasma protein

In 4 animals the total plasma proteins were estimated after injection of coagulase daily for 8 days. The total protein value fell rapidly with the fibrinogen after intravenous coagulase 1 injection, but the fall was much greater (0·8 to 1·4 g./100 ml.) than could be accounted for by removal of fibrinogen alone. Thereafter the total protein value slowly returned towards the original level (*e.g.*, Table I, rabbit F6). The albumin values remained constant and the fall was largely accounted for by a general decrease in globulins, particularly in the gamma globulin fraction.

Inhibition of coagulase in vitro

The coagulase inhibitory titre of the serum of 5 normal rabbits ranged from 1 in 16 to 1 in 64, but in immune rabbits it was 1 in 1000 or more. This inhibition refers to titrations against both coagulase 2 and the homologous coagulase 3.

Inhibition of coagulase in vivo

Graded doses of coagulase 2 were injected intravenously into rabbits and it was found that the fall in fibrinogen was roughly proportional to the amount of coagulase injected (Table II). With 0·125 mg. of this preparation in normal rabbits approximately 60–70 per cent of fibrinogen was removed from the blood in 30 min. When the same amount was injected into actively immunised rabbits

TABLE II.—*Percentage of Fibrinogen Removed from Blood in Coagulase Immune and Non-immune Rabbits at 30 min. after Intravenous Injection of Coagulase 2*

Rabbits E43, C8, and F7 were actively immunised against coagulase.

Rabbit No.	Amount of coagulase 2 (mg.)	Fibrinogen level (mg./100 ml.)		Percentage of fibrinogen removed	Anti-coagulase titre (reciprocal) of serum
		Zero	30 min.		
F28	1·0	210	30	86	64
F20	0·5	170	40	77	32
F19	0·25	310	92	70	—
F36	0·125	270	104	61	—
F17	0·125	338	120	66	16
F27	0·125	300	87	71	64
F23	0·125	435	120	72	32
E43	0·125	255	217	15	1000
C8	0·125	265	190	48	1000
F7	0·125	450	320	29	2000

whose sera inhibited coagulase, the quantity of fibrinogen removed was very much less (15, 29 and 48 per cent respectively).

Histological evidence of intravascular clotting

No macroscopic abnormality was seen in the organs of rabbits killed by, or after, the intravenous injection of coagulase. Microscopically, however, numerous and widespread intravascular lesions were evident. The lungs were the most severely affected organs and when the injections were given very rapidly and death occurred within 5 or 10 min., few lesions were seen in other viscera. If the coagulase was administered more slowly (15–20 min.) the intravascular lesions were readily seen in other organs such as the kidneys and liver but were never as numerous as in the lungs.

The lesions consisted of intravascular deposits giving the typical staining reactions of fibrin. These deposits occluded capillaries and arterioles mainly, and, to a lesser extent small and moderate sized arteries. The deposits were formed of multiple strands of fibrin arranged parallel to one another and to the long axis of the vessel which they occluded (Fig. 1). They were entirely intravascular, occasionally enmeshing small numbers of red cells. No evidence of damage to the vessel walls was seen.

In the lungs an enormous number of fibrin deposits occluded the majority of the vessels of capillary size (Fig. 2) and death appeared to be due to acute right heart failure following mechanical blockage of the pulmonary vasculature.

Effect of coagulase on mice

Doses of up to 2 mg. of the very potent coagulase 2 were injected intravenously into pairs of mice. After one hour one of each pair was killed. The remaining mice survived apparently unaffected by the injection and no evidence of undue bleeding was noted. Microscopical examination of the organs of the mice which were killed failed to show any evidence of intravascular clotting, such as was seen in the rabbit, or any other abnormality.

DISCUSSION

The present experiments give direct evidence that coagulase causes clotting *in vivo*. The failure of some of the previous attempts to show this action may be related to the route by which coagulase was introduced, *i.e.*, subcutaneous, intracutaneous and intrapleural (Menkin and Walston, 1935; Fisher, 1936) and to the low potency of the coagulase used. Although Fisher used the intravenous route the coagulase injected was comparatively weak. Lack and Wailling (1954) injected intrapleurally into rabbits plasma along with a potent purified coagulase similar to that used in the present work. They attributed their failure to show clotting *in vivo* to the action of staphylokinase present in the preparation of coagulase itself.

Since coagulase will clot heparinised or oxalated blood *in vitro*, it might have been expected that blood drawn shortly after the injection of massive doses of coagulase would clot despite the presence of an anticoagulant. The fact that it did not do so suggests the absence of active coagulase; its dilution, neutralisation by antibodies, and fixation in the tissues may account for this.

In mice no histological changes, such as were found in rabbits, followed the intravenous injection of coagulase ; the explanation of this lies probably in the relative deficiency of coagulase-reacting factor of mouse blood, approximately one hundredth that of rabbit blood (Tager and Hales, 1948*b*).

The coagulase preparations used, although purified, were not pure. The possibility, however, that some fraction other than coagulase was responsible for the *in vivo* clotting action is remote. Coagulase has a known *in vitro* clotting activity and therefore it seems reasonable to assume that both the fall in fibrinogen level and the intravascular fibrin deposits are the results of the action of coagulase itself.

SUMMARY

Potent purified coagulase injected intravenously into rabbits produced fibrinogen depletion and intravascular clotting.

The fibrinogen level fell precipitously after injection, then rose rapidly, reaching twice the original level at 48 hr., and returned to normal in a week. The fall in fibrinogen was much less in rabbits immunised against coagulase.

No significant fall in fibrinogen was observed when coagulase, inactivated by autoclaving, was injected into normal rabbits.

Following intravenous coagulase injection into rabbits multiple fibrin deposits occluded many capillaries and small vessels : they were most marked in the lungs but occurred also in the liver and kidneys.

In mice no changes were observed after the intravenous injection of coagulase.

We are indebted to Dr. E. S. Duthie (Royal South Hants. and Southampton Hospital) for the generous gift of purified coagulase and to Dr. E. B. Hendry (Western Infirmary, Glasgow) and his staff for all the biochemical estimations.

REFERENCES

Barry, A. P., Feeney, J. K. and Geoghegan, F. J.—(1955) *Brit. med. J.*, ii, 12.
Boake, W. C.—(1956) *J. Immunol.*, **76**, 89.
Cruickshank, R.—(1937) *J. Path. Bact.*, **45**, 295.
Fisher, A. M.—(1936) *Johns Hopk. Hosp. Bull.*, **59**, 393.
Gornall, A. G., Bardawill, C. J. and David, M. M.—(1949) *J. biol. Chem.*, **177**, 751.
Hale, J. H. and Smith, W.—(1945) *Brit. J. exp. Path.*, **26**, 209.
Kaplan, M. H. and Spink, W. W.—(1948) *Blood*, **3**, 573.
Lack, C. H. and Wailling, D. G.—(1954) *J. Path. Bact.*, **68**, 431.
Lominski, I.—(1949) *J. gen. Microbiol.*, **3**, 9.
Idem and Roberts, G. B. S.—(1946) *J. Path. Bact.*, **58**, 187.
Menkin, V. and Walston, H. D.—(1935) *Proc. Soc. exp. Biol., N.Y.*, **32**, 1259.
Much, H.—(1908) *Biochem. Z.*, **14**, 143.
Rammelkamp, C. H., Badger, G. F., Dingle, J. H., Feller, A. E. and Hodges, R. G.—(1949) *Proc. Soc. exp. Biol., N.Y.*, **72**, 210.
Smith, D. D. and Johnstone, J. M.—(1956) *Nature*, **178**, 982.
Smith, W., Hale, J. H. and Smith, M. M.—(1947) *Brit. J. exp. Path.*, **28**, 57.
Tager, M.—(1954) *Bull. N.Y. Acad. Med.*, **30**, 475.
Idem and Hales, H. B.—(1948*a*) *J. Immunol.*, **60**, 1.—(1948*b*) *Yale J. Biol. Med.*, **21**, 91.

THE TITRATION OF POLIOMYELITIS NEUTRALIZING ANTIBODIES

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THE two tests most widely employed in titrating poliomyelitis neutralizing antibodies are the cytopathic and the metabolic-inhibition or colour test (Youngner, 1955). In both tests, serum dilutions are mixed with a suspension of one of the types of poliomyelitis virus, usually containing 100 $TCID_{50}$, and allowed to react at room temperature for one hour. In the cytopathic test, the mixtures are then added to prepared cell cultures which are examined microscopically after 5 to 7 days' incubation and the highest dilution of serum neutralizing the virus determined. In the colour test, a cell suspension is added to the serum-virus mixtures and the end-point of neutralization determined by noting the colour change of the phenol red indicator after the cultures have been incubated for 5 to 7 days. Both tests may be done either in test tubes or on plastic trays (Barski and Lepine, 1954; Salk, Youngner and Ward, 1954; Melnick and Opton, 1956).

In our hands these methods have not proved entirely satisfactory for determining antibody responses to formalinized poliomyelitis vaccine either in monkeys or in humans. Irregular results were obtained when the same sera were titrated on different occasions or by different workers and it was considered that the irregularity was due to incomplete neutralization of virus by antibody. Preliminary experiments showed that poliomyelitis viruses were neutralized slowly and that it was necessary, in order to obtain complete neutralization, to employ a longer period and a higher temperature than " one hour at room temperature ". More consistent results were obtained when serum-virus mixtures were incubated at 37° for 3 hr. before adding them to prepared cell cultures and it was on the basis of this finding that these conditions were adopted for routine titrations by the Biological Standards Control Laboratory (Medical Research Council Reports, 1957). As this test differs in some important details from those most commonly used, a more detailed description of it is given here. The test has also been compared with the metabolic-inhibition test. Also included in this account are the results of a number of experiments indicating the influence of time and temperature on the neutralization of poliomyelitis viruses by antibody.

MATERIALS

Test viruses.—The three virus strains employed in the production of British poliomyelitis vaccine were used in titrating poliomyelitis antibodies, namely the attenuated Brunenders strain for type 1, MEF1 for type 2 and Saukett for type 3. Fluid from infected monkey kidney cultures was centrifuged at 2500 r.p.m. for 20–30 min. and used as virus pools. These were kept frozen at — 30° in small aliquots and diluted for use in Parker 199 medium so that the

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test dose of 0·25 ml. contained approximately 100 $TCID_{50}$. A virus control titration, in which the test dose was diluted 1/10, 1/100 and 1/1000, was included in all tests. Virus pools have shown no loss of titre over periods of up to two years.

Reference and test sera.—The three British reference poliomyelitis sera were used for control titrations. The sera were prepared in monkeys and aliquots of each were dried in ampoules containing 10 units (to be published). They were reconstituted by adding 1 ml. of Hanks balanced salt solution (BSS) to each ampoule which was held at 37° for 15 min. to allow the dried powder to dissolve completely. The reconstituted serum was transferred to a sterile 10 ml. measuring cylinder and the ampoule washed out two or three times with more diluent to ensure the removal of all the serum. These washings were added to the cylinder and the volume made up to exactly 10 ml. to give a serum dilution of 1/10 containing 1 unit-/ml. Dilutions of the reference sera were kept at 4° and always used within one week of preparation.

Samples of test sera were inactivated at 56° for 30 min., and stored at − 10° if not required immediately.

Cell cultures and suspensions.—Trypsinized monkey kidney cell cultures were prepared in 6 × $\frac{5}{8}$ in. test tubes, as described previously (Biological Standards Control Laboratory Report, 1957). Before the cultures were used, the old 199 medium containing 5 per cent horse serum was removed and the cells in each tube washed once with 1 ml. of BSS followed by a re-feeding with 1 ml. of 199 medium containing 0·07 per cent bovine albumin (Armour Fraction V) and 0·2 per cent sodium bicarbonate.

Cell suspensions were prepared from trypsinized monkey kidney cell cultures grown in 500 ml. medical flats ; 0·02 per cent versene solution was added for 30 min., at 37° to remove the cells from the glass. The resulting suspension was centrifuged at 1000 r.p.m. for 10 min. and the cell deposit resuspended in BSS containing 0·5 per cent lactalbumin hydrolysate, 0·1 per cent glucose and 0·26 per cent sodium bicarbonate, to give 130,000 cells/ml.

EXPERIMENTAL

The B.S.C.L. Cytopathic Neutralization Test

Serial dilutions of the reference and test sera are made in BSS. Human sera are usually diluted 4-fold and the monkey and the reference sera (1 unit/ml.) 2-fold. To each dilution, an equal volume of virus suspension is added and, after thorough shaking, the mixtures are incubated at 37° for 3 hr. ($3\frac{1}{2}$ hr. in an air incubator). The mixtures are then added to previously prepared monkey kidney cultures or held overnight at 4° and added to the cultures next day. Each culture tube receives 0·5 ml. of mixture ; for each virus-serum dilution four culture tubes are inoculated when titrating the reference sera and two tubes when titrating the test sera. The tubes are incubated at 37° in roller drums, read at 6 days and the dilution of serum neutralizing virus in 50 per cent of cultures determined. Titres are expressed as the dilution of serum in the serum-virus mixtures before they are added to the cultures.

The test is used routinely in this laboratory for titrating both human and animal sera. In each set of tests a titration of the appropriate reference serum is always included and the results of a number of these titrations made over a period of 12 months are given in Table I.

Effect of Time and Temperature on the Neutralization of Virus

Three groups of tests were made using the 3 reference and 6 human sera. Each group contained the homotypic reference, one human serum of low titre and another of high titre to the test virus. In these experiments 2-fold dilutions were made of the reference sera and 4-fold dilutions of the human sera. To each dilution an equal volume of the appropriate virus suspension was added. Each group of tests was then divided into three subgroups, one being incubated at 37°, another at

TABLE I.—*Results of Repeated Titrations of the British Reference Poliomyelitis Sera*

Serum titre	Number of titrations giving titre to: Type 1	Type 2	Type 3
240	1	—	—
254	1	—	—
320	15	—	—
404	12	—	—
480	36	—	—
508	6	—	—
640	1	15	3
806	—	9	9
960	—	20	26
1016	—	5	7
1280	—	7	19
1610	—	4	4
1920	—	—	1
Total number of titrations	72	60	69
Maximum variation	2·7	2·5	3·0
Geometric Mean titre	424	908	855

room temperature (23–24°) and the third at 4°. At different times 1·5 ml. aliquots of the reference serum-virus mixtures and 2·0 ml. of the human serum-virus mixtures were removed and transferred in 0·5 ml. volumes to three and four culture tubes respectively. The tubes were incubated at 37° in roller drums and read after 6 days. The results are given in Table II.

In the tests made at 37°, there was a rise in titre of all 9 sera with time of contact of serum and virus. In some, neutralization of virus appeared to be complete at this temperature after 3 hr. or even sooner, while in others, especially those of types 2 and 3, there was some indication that neutralization continued to occur between 3 and 5 hr. In the tests made at room temperature a similar increase in titre with time of contact was observed, although the values at the different times were less regular and generally lower than those in the tests made at 37°. A rise in titre with time also occurred in the tests made at 4° but the increase was slower than those at the higher temperatures and only occasionally, even after 24 hr. contact, did the titre reach the highest level obtained in the tests made at 37°.

The effect of time and temperature on serum titres was also evident from the results of tests in which a number of human and monkey sera were titrated after being held in contact with virus for 3 hr. at 37° and also for one hour at room temperature. Titrations of the human sera by these two methods were done in parallel and the titres were thus strictly comparable but titrations of the monkey sera were done on separate occasions. The results are summarized in Table III and it is noted that the titres obtained using the higher temperature and longer period of contact were always higher than the others.

A further experiment, the results of which are shown in Table IV, was made with more monkey and human sera, in which the titres obtained after incubating serum-virus mixtures at 37° for 3 hr. were compared in the same test with those obtained after incubating at 37° for 5½ hr. Again, as in the experiment reported in Table II, in some of the titrations neutralization was complete at 3 hr. but with

TABLE II.—*The Influence of Time and Temperature on the Neutralization of Poliomyelitis Virus by Antibody*

Virus type	Serum	Temperature of neutralization	Antibody titre after time of contact of serum and virus (hours) 0·5	1	2	3	5	24
1	Reference	37°	132	214	360	360	360	—
		23°	130	214	214	276	214	—
		4°	—	—	214	—	214	360
	Human A	37°	<12	12	22	44	32	—
		23°	<12	12	16	30	24	—
		4°	—	—	24	—	24	24
	Human B	37°	128	354	256	354	512	—
		23°	194	128	256	128	194	—
		4°	—	—	194	—	194	256
2	Reference	37°	576	—	488	960	2,300	—
		23°	228	576	—	728	1,920	—
		4°	—	—	364	—	364	1,152
	Human E	37°	48	92	64	192	284	—
		23°	64	96	64	192	284	—
		4°	—	—	64	—	96	228
	Human F	37°	356	324	—	8,192	11,628	—
		23°	252	256	252	5,824	11,628	—
		4°	—	—	256	—	4,096	8,192
3	Reference	37°	664	916	728	960	1,028	—
		23°	484	484	364	960	728	—
		4°	—	—	228	—	564	564
	Human C	37°	160	140	224	224	448	—
		23°	224	224	112	320	76	—
		4°	—	—	160	—	212	156
	Human D	37°	4,096	4,096	4,096	5,780	16,384	—
		23°	2,896	—	5,780	2,896	5,780	—
		4°	—	—	2,048	—	4,096	8,192

TABLE III.—*Comparison of Poliomyelitis Antibody Titres obtained after Neutralization at Room Temperature for* 1 *hr. and at* 37° *for* 3 *hr.*

Serum	Number of sera tested	Virus type	Geometric mean titre after neutralization at: 24° for 1 hr.	37° for 3 hr.
Human	8	1	38	148
	6	2	22	87
	10	3	128	512
Monkey	22	1	26	39
	21	2	12	89
	21	3	25	32

TABLE IV.—*Comparison of Poliomyelitis Antibody Titres obtained after Neutralization at 37° for 3 hr. and 5½ hr.*

Serum		Antibody titre to: Type 1 after		Type 2 after		Type 3 after	
		3 hr.	5½ hr.	3 hr.	5½ hr.	3 hr.	5½ hr.
Monkey	A	<4	<4	256	256	645	1024
	B	64	64	256	256	64	102
	C	32	32	25	40	<8	<8
	D	8	16	256	1024	645	645
	E	16	32	<8	<8	<8	<8
	F	—	—	645	1024	161	256
Human	G	1024	2048	40	102	1024	645
	H	8	8	2580	4096	645	1626
	I	1024	1024	8	8	161	256
	J	<8	<8	256	1024	1024	2580
	K	1024	512	406	6405	1626	1626
	L	256	64	645	1626	161	256
	M	2048	2048	—	—	256	406
	N	—	—	—	—	40	64

others, especially those of types 2 and 3, neutralization continued to occur after 3 hr.

Comparison of the Cytopathic and the Colour Tests

In titrating serum by the colour test, 4-fold dilutions of serum were made in BSS and to each dilution an equal volume of virus suspension was added. The mixtures were held either at room temperature for 1 hr. or at 37° for 3 hr. and then transferred, in 0·5 ml. volumes, to 3 × ½ in. test tubes, four tubes being used for each virus-serum dilution. Monkey kidney cell suspension, in 0·5 ml. volumes, was added to the tubes which were afterwards "sealed" with 0·5 ml. of liquid paraffin. The tubes were then incubated at 37° for 6 days. If, however, before 6 days there was a tendency for the mixtures to become acid, 0·02 ml. of 0·05 N NaOH solution was added to each tube. On the sixth day, one drop of 10 per cent glucose solution in distilled water was added and 2 days later the colour changes of the phenol red indicator recorded. The addition of glucose was found to produce a much clearer end-point. Serum titres were calculated as in the other tests.

A comparison of the colour test with the cytopathic test was made by titrating type 1 antibody in sera taken from 8 subjects 14 days after receiving the second of two doses of poliomyelitis vaccine. In both tests, serum dilutions were left in contact with virus for 3 hr. at 37° and also for one hr. at room temperature. The cells used in preparing both the cell cultures and the suspension were derived from the same monkey in order to exclude any variation in sensitivity to virus. The results are given in Table V and it is evident that, in both tests, time and temperature of contact had an effect on the titre. With each of the 6 positive sera, higher titres were obtained when the test was made at 37° for 3 hr. It is also evident that the cytopathic test gave readings similar to those of the colour test when the conditions of neutralization were the same.

Comparative titrations were also made with sera taken from 3 subjects at various times after the first injection of poliomyelitis vaccine. Three methods of titration were compared, the cytopathic and colour tests, using one hr. at room temperature

TABLE V.—*Comparison of Type 1 Poliomyelitis Antibody Titres in Human Sera obtained by Cytopathic and Colour Neutralization Tests*

Serum No.	Antibody titre after neutralization at: 23° for 1 hr. Cytopathic test	23° for 1 hr. Colour test	37° for 3 hr. Cytopathic test	37° for 3 hr. Colour test
10	<4	<4	<4	<4
11	79	203	325	512
12	128	128	512	512
13	32	32	128	128
14	8	6	8	12
15	8	12	20	32
16	<4	<4	<4	<4
17	12	12	32	32

TABLE VI.—*Comparison of Type 1 Poliomyelitis Antibody Titres obtained in Cytopathic and Colour Neutralization Tests*

Human subject	Number of days after one dose of polio vaccine when serum taken	Antibody titre after neutralization at: 23° for 1 hr. Cytopathic test	23° for 1 hr. Colour test	37° for 3 hr. Cytopathic test
M	0	128	128	512
	3	128	128	512
	8	384	325	1318
	12	2048	2048	3311
	14	—	1318	2048
F	0	<4	<4	<4
	3	<4	<4	<4
	6	—	<4	<4
	13	3	8	8
W	0	<4	<4	<4
	3	<4	<4	<4
	8	—	12	20
	15	8	12	32

for neutralization, the conditions most commonly used, and the B.S.C.L. test using 37° for 3 hr. The results, given in Table VI, show that 7 of the 8 positive sera gave higher titres by the B.S.C.L. test than by the other methods.

DISCUSSION

Although much work has been published on the neutralization of viruses by their antisera (see Kjellen, 1957), little has been done on the neutralization of the poliomyelitis viruses. The techniques used in the estimation of poliomyelitis antibodies have usually been chosen somewhat arbitrarily as far as the time and temperature of neutralization of virus by serum have been concerned. Dulbecco, Vogt and Strickland (1956) studied the neutralization of type 1 poliomyelitis virus by two hyperimmune sera, one prepared in a monkey and the other in a rabbit. They found that neutralization was completed in a short time and that dissociation of the complex was negligible. Recently Gard (1957), working with a single

guinea-pig serum, showed that the complete neutralization of type 1 poliomyelitis virus was a slow process and dependent on both time and temperature. This has been our experience also when testing both human and monkey sera for poliomyelitis antibodies produced by vaccination with formalinized vaccine. With type 1 virus, 3 hr. at 37° seemed to be adequate for serum titres to become stabilized and since all the preliminary experiments were done with this type, 3 hr. was chosen as a suitable period of contact. With types 2 and 3 viruses, however, neutralization by some sera may require a longer period. As a routine procedure, periods of incubation longer than 3 hr. may not result in a significantly better reproducibility to warrant an extension of the time of neutralization. In order to resolve this problem tests are now being made to determine whether, by extending the period of incubation to $5\frac{1}{2}$ hr., a higher degree of reproducibility is obtained. It is considered that $5\frac{1}{2}$ hr. is a suitable period to investigate, since thermal inactivation of virus during this period at 37° is negligible (Youngner, 1957) but beyond this time it could give rise to spurious increases in serum titres.

It might be more advantageous for serum titres to be expressed in terms of units than in dilutions. If reference to a standard serum is to be made, however, the test must be such that neutralization of virus by serum attains a stable state. Under these conditions reference may then be made irrespective of the details of the test. This suggestion will be the subject of a further communication.

Although it has been shown that neutralization of virus by serum is more complete at 37° for 3 hr. than at room temperature for 1 hr., the former conditions may not necessarily be more suitable for the serological diagnosis of poliomyelitis. Sabin (1957) has recently suggested that antibody produced early in poliomyelitis infection is less avid than that produced later. A rising antibody titre during infection may thus be more easily demonstrated by a less sensitive test. Nevertheless, for the titration of antibody after vaccination the most sensitive method available is preferable.

SUMMARY

In titrating poliomyelitis neutralizing antibodies by either the cytopathic or colour tests, the time and temperature of contact of serum and virus before adding the mixtures to cell cultures are important factors. Incomplete neutralization occurs when serum and virus are held at room temperature for 1 hr., which are the conditions most commonly employed in titrating poliomyelitis antibodies.

More complete neutralization occurs when serum-virus mixtures are held at 37° for 3 hr., although in some titrations, mainly those with types 2 and 3, there is a tendency for further neutralization to occur after this time.

REFERENCES

Barski, G. and Lepine, P.—(1954) *Ann. Inst. Pasteur.*, **86**, 693.
Biological Standards Control Laboratory Report.—(1957) *Brit. med. J.*, ii, 124.
Dulbecco, R., Vogt, M. and Strickland, G. A.—(1956) *Virology*, **2**, 162.
Gard, S.—(1957) *Arch. ges. Virusforsch.*, **7**, 449.
Kjellen, L.—(1957) *Ibid.*, **7**, 307.
Medical Research Council Reports.—(1957) *Brit. med. J.*, i, 366.—*Ibid.*, ii, 1207.
Melnick, J. L. and Opton, E. M.—(1956) *Bull. World Hlth Org.*, **14**, 129.
Sabin, A. B.—(1957) *Spec. Pub. N.Y. Acad. Sci*, v, 113.
Salk, J. E., Youngner, J. S., Ward, E. N.—(1954) *Amer. J. Hyg.*, **60**, 214.
Youngner, J. S.—(1955) *Ann. N.Y. Acad. Sci.*, **61**, 774.—(1957) *J. Immunol.*, **78**, 282.

STUDIES OF A COXSACKIE VIRUS ANTIGENICALLY RELATED TO ECHO 9 VIRUS AND ASSOCIATED WITH AN EPIDEMIC OF ASEPTIC MENINGITIS WITH EXANTHEM

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In recent years, and especially in 1956, outbreaks have occurred in which cases with maculopapular rash resembling rubella and often associated with aseptic meningitis have been seen. From patients in these outbreaks viruses have been recovered in tissue culture and have been found to possess pathogenic properties for suckling mice resembling those of Coxsackie A viruses (Tyrrell and Snell, 1956; Lennartz, Maass and Kersting, 1957). These viruses are however neutralized by serum prepared against the ECHO 9 virus (Enteric Cytopathogenic Human Orphan virus type 9) (McLean and Melnick, 1957; Boissard, Stokes, Macrae and MacCallum, 1957; Quersin-Thiry, Nihoul and Vekking, 1957). The ECHO 9 prototype virus was isolated in tissue culture from the faeces of a normal child and is non-pathogenic for suckling mice (Committee on ECHO viruses, 1956; Ramos-Alvarez and Sabin, 1954).

We have studied in detail the physical and general biological properties of a number of these recently isolated strains and the results are presented in this paper. Clinical and epidemiological aspects of the disease in the Sheffield area associated with the viruses have been published elsewhere (Tyrrell, Lane and Snell, 1958).

MATERIALS AND METHODS

Tissue cultures.—Roller tube cultures were used almost exclusively. Trypsinized cultures of monkey and human kidney were used mainly and the methods were based on those of Melnick (1955). Other trypsinized cultures included human amnion (Zitcer, Fogh and Dunnebacke, 1955) and human Fallopian tube epithelium (Tyrrell—unpublished) and the continuous cultures of human liver cells (Chang, 1954) and HeLa cells (Scherer, Syverton, and Gey, 1953). Plasma clot cultures of human lung and of hamster and mouse leg fragments were made by the method of Robbins, Weller and Enders (1952). Trypsinized mouse, guinea-pig and chick embryo kidney cultures were prepared using the modified procedure of Heath (to be published).

Animal inoculations and titrations were carried out as described elsewhere (Tyrrell and Cameron, 1957). All animals were observed for at least two weeks.

Storage of infectious material.—Throat, mouth and nose swabs were placed in a few ml. of sterile skim-milk-saline. Faeces, throat swabs and culture fluids were stored in screw capped bottles at about − 26° or occasionally at − 70°.

Preparation of immune sera.—Mice and hamsters were immunized by giving 2 injections of 0·2–0·5 ml. of culture fluid intraperitoneally about one week apart. The animals were

* Member of the External Scientific Staff of the Medical Research Council.

bled from the heart one week after the last injection. The immunization of rabbits was carried out as described for adenoviruses (Balducci, Zaiman and Tyrrell, 1956). All sera were separated aseptically, stored at − 26° and inactivated at 56° for 30 min. shortly before use.

Complement fixation test.—This was done on plastic plates by a method identical with that used for adenovirus work (Balducci *et al.*, 1956).

Neutralization tests in roller tube cultures using 50 TCD_{50} of virus were performed as described for poliomyelitis virus (Tyrrell, Keeble and Wood, 1956). Unless otherwise stated, all negative sera were tested at a dilution of 1/8 in an inoculum of 0·5 ml. in the neutralization test and at a dilution of 1/4 in the complement fixation test.

Histological methods.—Trypsinized kidney cells were cultivated on coverslips measuring 9 × 32 mm. lying in stationary tubes. Suckling animals were fixed whole after incising the scalp and abdomen and parasagittal sections were examined. All material was fixed in Bouin's fluid and stained with haematoxylin and eosin.

Viruses.—Strains isolated in Sheffield between August and November 1956 were used for most experiments. They are denoted by serial numbers except for the prototype strain—Haynes—referred to earlier (Tyrrell and Snell, 1956). Other strains generously supplied by other workers are listed below.

Q.E. from Dr. A. D. Macrae (see Crawford, Macrae and O'Reilly, 1956) isolated in 1954 in an epidemic in London of rash with abnormal C.S.F. *Ipswich* from Dr. G. P. B. Boissard (see Boissard, Stokes, Macrae and MacCallum, 1957) isolated in an epidemic of aseptic meningitis with occasional rash. *Bourn* and *Dodds* were supplied by Dr. D. M. McLean and isolated from epidemics with aseptic meningitis occurring near Cambridge, England (McLean and Melnick, 1957). *Pi 3* and *Sas. 3* were isolated by Professor Geo. Rita from cases of aseptic meningitis occurring in Italy in 1955 (Rita, Russi and Fischer-Fantuzzi, 1956). *1043* was received from Professor E. Pette of Hamburg. It is one of 104 similar strains isolated during a widespread epidemic of meningitis and exanthem in Germany in 1956 (Lennartz *et al.*, 1956). *Clegg* was supplied by Dr. A. J. Rhodes and isolated in 1956 from an epidemic of aseptic meningitis and exanthem occurring in Toronto, Canada (LaForest, McNaughton, Beale, Clarke, Davis, Sultanian and Rhodes, 1957). The above viruses will at times be referred to as Coxsackie ECHO 9 viruses. ECHO 9 prototype strain (Hill) and immune serum were supplied by Dr. A. D. Macrae.

EXPERIMENTAL

The isolation of viruses in tissue cultures

In all we examined about 130 specimens of faeces, nose and throat swabs and C. S. F. from cases in the epidemic and from other subjects. From these 33 strains of virus were isolated and also 4 strains of poliomyelitis type I and one strain of adenovirus. The inoculum consisted of a total of 1·5 ml. of supernatant from a 10 per cent faecal suspension or of supernatant from the milk saline from the bottle containing a nose and throat swab. The faeces suspension was removed within 8 hr. after inoculation. Specific cell degeneration was seen in cultures between 2 and 6 days after inoculation with throat swab material. In cultures inoculated with faeces degeneration occurred between 1 and 6 days after inoculation, but it was not always possible to distinguish it from non-specific toxic cell damage until a passage was performed. In every case a second or even third passage was performed but in all specimens from which virus was isolated degeneration was seen on the first passage. Most of the isolations were carried out using human kidney cells, but monkey kidney cells were apparently equally satisfactory and were used almost exclusively for titrations of virus and for many neutralization tests.

Cell degeneration and virus multiplication in tissue cultures

The results of a number of experiments on the growth of these viruses in tissue cultures are summarized in Table I. Almost all strains have been found to

TABLE I.—*Results of Inoculation of Virus (Strain 864) into Tissue Cultures*

Species	Type of culture and cell	Cell degeneration seen*	Titre of virus formed†
Human	Trypsin dispersed kidney	+	10^{6}
,,	,, ,, amnion	+	$10^{4.1}$
,,	,, ,, fallopian tube	+	$10>^{7.0}$
,,	,, ,, liver (Chang)	0	$10^{4.5}$
,,	,, ,, cancer (HeLa)	0	0
,,	Plasma clot embryo lung	+	$10^{6.1}$
Monkey	Trypsin dispersed kidney	+	$10^{6.5}$
Rabbit, guinea-pig, chick, mouse	,, ,, ,,	0	0
Mouse	Plasma clot, leg	0	$10^{0.9}$
Hamster	,, ,, ,,	0	0

* After inoculation of about 10^{5} TCD_{50} and less.
† After inoculation of 50–160 TCD_{50}.
All virus titrations done in monkey kidney cultures.

cause degeneration in human and monkey kidney cultures, but a few have been tested in only one of these systems. The degeneration usually starts as a focus of a few cells which tend to become spherical or polygonal with fine extensions of the cytoplasm. In the course of about 24 hr. these cells become completely rounded and converted to a mass of fine granules and the changes spread to the whole of the culture. The changes are less marked in stationary than in rolled cultures and there is a tendency in working with recently isolated strains and with stationary cultures for the changes to affect only a proportion of the cells. The surviving cells may then spread out and reform a complete cell sheet. These changes are illustrated in Fig. 1, 2 and 3. When the changes progress to complete destruction they are indistinguishable in fresh preparations from those produced by poliomyelitis. Stained cultures show changes like poliomyelitis too. There is an early coarsening of the chromatin pattern of the nucleus with the formation of an eosinophilic cytoplasmic mass which may appear to compress the nucleus (compare photomicrographs in Beale, Stevens, Davis, Stackiw and Rhodes, 1956 ; Harding, Harding, McLimans and Rake, 1956 ; Reissig, Howes and Melnick, 1956). Retraction of the cell margin and formation of " bubbles " and " beads " of cytoplasm attached to long threads are seen (Fig. 4) exactly like those described in poliovirus-infected cells (Buckley, 1956). Finally the cells become rounded and deeply eosinophilic with a small irregular basophilic area instead of a nucleus.

Human embryo lung fragments were cultured in plasma clots and degeneration of the fibroblast outgrowth occurred between 3 and 9 days after inoculating each of 12 strains. The inoculum was 0·5 ml. of fluid from the human kidney cell culture in which the virus was isolated. Control cultures inoculated with non-infectious culture fluids did not degenerate. Rounded cells were first noticed near the fragment of tissue (Fig. 5) and cell degeneration then spread outwards into the sheet of fibroblasts (Fig. 6). This has been noticed with Coxsackie viruses and the Neva agent ECHO type 16 (Weller, Robbins, Stoddard and Florentino, 1953 ; Neva and Enders, 1954). It was noted that the alveoli which could be clearly seen in the tissue fragments in uninfected cultures were no longer visible when degeneration of the outgrowth was still at an early stage. Sections of fragments of tissue removed shortly after inoculation showed that the alveoli were filled with eosinophilic debris and the degenerate cells shed from the lining

membrane. Many fibroblasts in the supporting tissue became degenerate until finally no normal cells or structures could be recognized (Fig. 7 and 8). Virus-infected human amnion and Fallopian tube cell cultures also degenerated, but the cell changes were not studied in detail. Numerous experiments in which serial passage was performed in monkey and human kidney cultures indicate that the virus multiplies in these cultures. In some experiments, shown in Table I, cultures were inoculated with 50–160 monkey kidney TCD_{50} of a virus (strain 846) which had had one pass in human kidney and one in monkey kidney cell cultures. The medium was collected two days later and titrated in monkey kidney cultures. Virus multiplication apparently occurred in amnion and Fallopian tube cultures. Some multiplication also occurred in human liver cells maintained in continuous culture. There was no cell degeneration and serial passage was not successful after the second pass. There was no multiplication in HeLa cells and no degeneration was seen in these or any of the other animal cell cultures used. It is probably significant that in one of two experiments a little virus was found in the fluid from plasma clot cultures of fragments of suckling mouse leg tissues, but virus multiplication was not conclusively demonstrated.

Disease and virus multiplication in experimental animals

Undiluted tissue culture fluid of strain 846 ($10^{4.8}TCD_{50}$) was inoculated subcutaneously and intracerebrally to adult mice and suckling guinea-pigs, and *via* the amniotic, allantoic and yolk sac cavity into embryonated eggs. In all these there was no sign of disease or gross pathological lesions. Suckling hamsters became feeble and ataxic 4 days after intracerebral and subcutaneous inoculation of virus but did not die; the muscle contained about $10^{7}TCD_{50}$/g. 3 days after inoculation. Suckling mice were inoculated subcutaneously and, intracerebrally, with the 846 Haynes, and other strains. The animals became ill with ataxia and increasing muscular weakness like that seen in Coxsackie A virus infections. Paralysis and death usually occurred between 4 and 12 days after inoculation, and infected muscle contained about $10^{8}TCD_{50}$/g. In many instances mice became paralysed and then apparently recovered completely. It was found that the age of the mice and the route of inoculation affected the outcome. In mice aged 1 to 2 days more effect was seen after intracerebral inoculation, as is shown in Table II.

TABLE II.—*Results of Inoculation of Virus (Strain Haynes) to Suckling Mice*

Age of mice (days)	Route of inoculation	Number of mice	
		Paralysed†	Dead
1–2	Intracerebral	12/14*	11/14
	Subcutaneous	4/14	2/14
4–5	Intracerebral	9/16	9/16
	Subcutaneous	15/23	12/23
9	Subcutaneous	3/10	3/10
14	Intracerebral + subcutaneous	0/7	0/7

† Mice found dead are included in the total of mice paralysed.
* Numerator = number affected. Denominator = number inoculated. A pool of virus obtained at 4th pass in human kidney cultures was used; it was inoculated undiluted.

Mice aged 14 days and older were resistant when inoculated by all routes. Mice aged 1 to 3 days were used for all other experiments. In a few instances up to 4 serial passages were readily carried out and disease was seen at each passage. A few experiments suggested that 4 rapid serial passages in monkey kidney cultures did not decrease the pathogenicity of strains which killed 100 per cent of suckling mice when first inoculated. On several occasions it was shown that when fluids which did not produce disease in suckling mice were passed in monkey kidney cultures the passage fluids were highly pathogenic for mice—presumably due to an increase in the concentration of virus present.

Histological examination of affected mice between 4 and 15 days after inoculation showed no abnormality except in striated muscle. In this tissue the sarcoplasm lost its angular outline on transverse section and was then destroyed. There was proliferation of sarcolemmal nuclei (Fig. 10) and little or no inflammatory reaction. Four paralysed mice which had recovered were examined between 17 and 33 days after inoculation. The muscle nuclei seemed rather prominent and in 2 mice basophilic masses were found in occasional muscle fibres (Fig. 11), but otherwise there was complete restoration of muscle stucture. These changes are identical with those seen in a mild Coxackie A virus infection (Gifford and Dalldorf, 1951). Fifteen out of 16 Sheffield strains, besides Haynes, inoculated into mice after one tissue culture passage, produced the characteristic paralysis and histological changes. In these experiments 84 out of 101 inoculated mice became paralysed and 50 out of 92 mice died (excluding those killed for histological examination). Two faecal suspensions containing virus and 4 samples of virus-free culture medium produced no paralysis or muscle lesions. The virus was

EXPLANATION OF PLATES

FIG. 1.—Normal human kidney cells. Unstained. × 85.

FIG. 2.—Human kidney cell culture 2 days after inoculation of strain Haynes. Many irregular or rounded degenerate cells. Unstained. × 85.

FIG. 3.—The same 3 days after inoculation of strain 811. Degenerate cells have fallen off the glass and the surviving cells have reformed a continuous sheet. In many cultures all the cells degenerated. Unstained. × 85.

FIG. 4.—Stained human kidney cells from a culture inoculated 2 days before with Haynes strain. Note deeply-stained rounded cells with threads of cytoplasm extending from the surface and 2 cells each with early nuclear changes and an eosinophilic mass in the cytoplasm. Haemotoxylin and eosin. × 445.

FIG. 5.—Early degenerative changes in a plasma clot culture of human embryo lung. Rounded cells are seen among the fibroblasts growing from the tissue fragment. Inoculated 7 days before with strain 839. × 85.

FIG. 6.—A late stage of the process seen in 5. The tissue fragment is crumbling up and almost all the fibroblasts are degenerate. Inoculated 7 days before with strain 854. × 85.

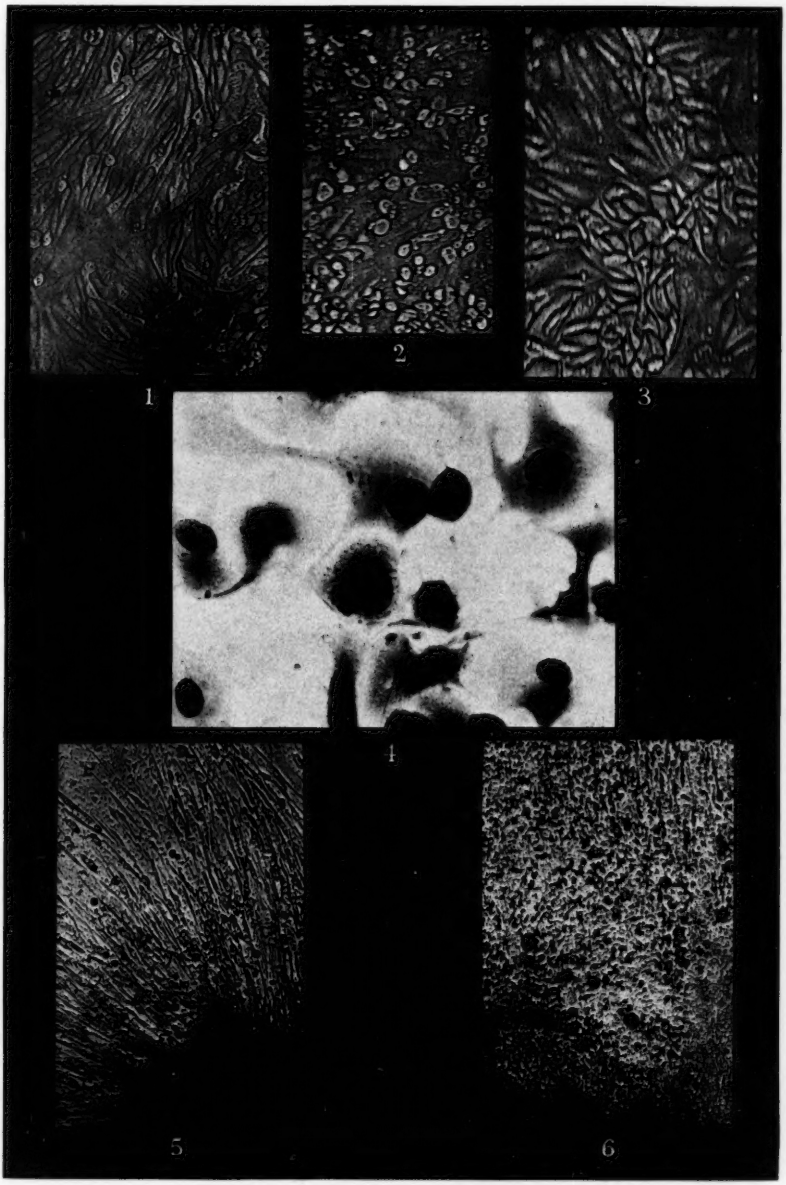
FIG. 7.—Stained section of a tissue fragment from an uninfected plasma clot culture of human embryo lung. H & E. × 80.

FIG. 8.—Stained section from a parallel culture to that shown in 7, infected 2 days previously with Haynes virus. Note the widespread cell destruction and collapse of the tissue. The degeneration of the fibroblast outgrowth resembled that shown in 5. H & E. × 80.

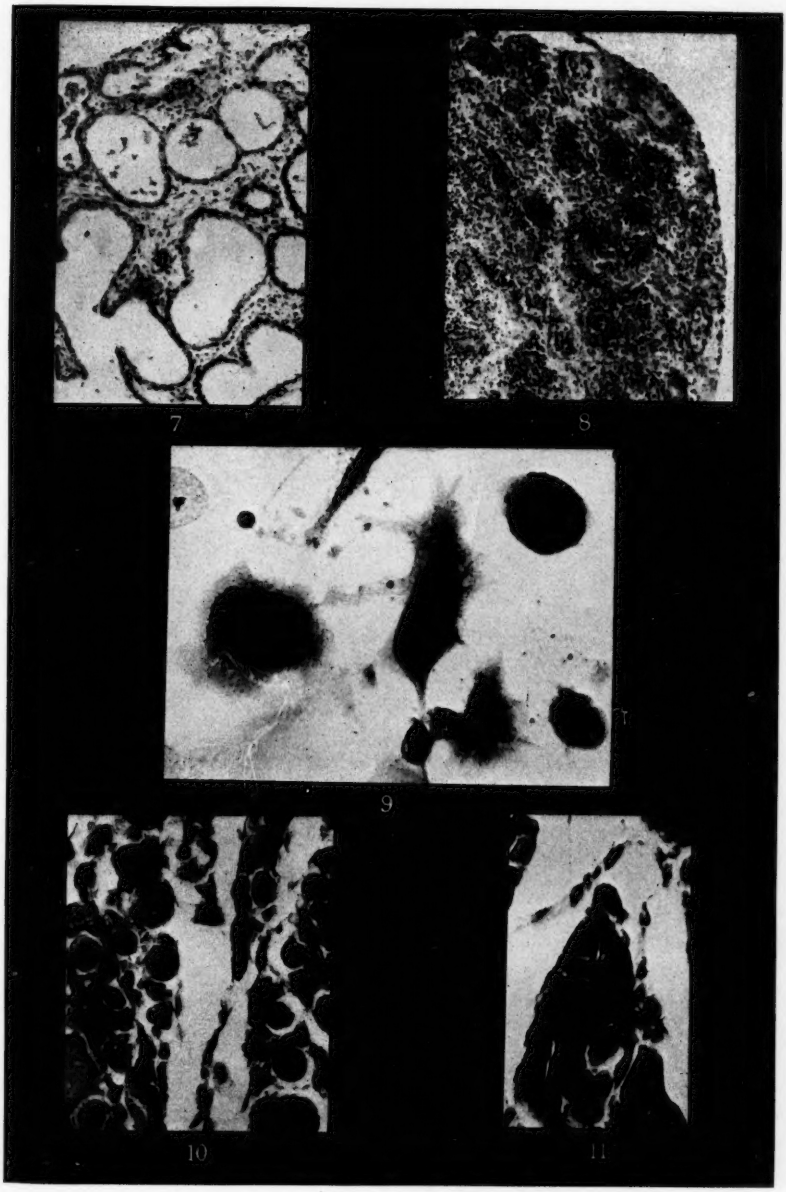
FIG. 9.—Monkey kidney cells inoculated with Coxsackie A9 virus, showing changes identical with those in 4. H & E. × 425.

FIG. 10.—Striated muscle from a severely paralysed suckling mouse inoculated with strain 834 and killed when apparently moribund 9 days after inoculation. Muscle fibres look rounded in outline and in some the sarcoplasm has disappeared and nuclei are apparently proliferating. H & E. × 80.

FIG. 11.—Striated muscle from a suckling mouse which became paralysed and then recovered completely 17 days after intracerebral inoculation with Haynes virus. Muscle structure has apparently been restored except for the presence of a basophilic mass of amorphous material. H & E. × 80.



Tyrrell, Clarke, Heath, Curran, Beswick and Wolman.



Tyrrell, Clarke, Heath, Curran, Beswick and Wolman.

inoculated into 6 monkeys. First a pair of rhesus monkeys was inoculated. One received undiluted culture fluid intracerebrally (about 10^7TCD_{50}) and the other intracerebrally and intraperitoneally; they developed slight fever a few days after inoculation and were killed 14 and 20 days after inoculation. Two rhesus and two cynomolgus monkeys were treated with cortisone and inoculated (with about 10^8TCD_{50}) into brain, spinal cord and muscle by the methods used in testing polio vaccine (Report, 1957). One of each pair of these animals was killed 6 days and 4 weeks after inoculation. Sections were made of all parts of the nervous system of these animals and stained with gallocyanin. No pathological changes were seen in nerve cells, apart from a little associated with the trauma of inoculation. One of the first two rhesus monkeys (that killed at 14 days) showed a lymphocytic meningitis, and the second two rhesus monkeys showed an arteritis of vessels on the surface of the brain stem, but it was not possible to be certain that these changes were directly due to the virus infection. The changes seen in no way resembled those seen after the inoculation of polioviruses, and there were no consistent or marked abnormalities in the muscles or viscera.

Physical properties of the virus

In order to assist in classifying this group of viruses some of the more readily measured physical properties were studied and the Haynes strain was used in almost all experiments.

Stability.—The stability of the virus when suspended in Hanks' saline at pH 7·4–7·8 was studied in the experiments summarised in Table III.

These show that the stability of the virus was very similar to that of poliovirus when held for 30 min. in a water bath at various temperatures. The virus was

TABLE III.—*Effect of Heating Virus (Strain Haynes) and Poliovirus (Strain Saukett) at Various Temperatures for 30 Minutes*

	Titre of virus after treatment at				
	Room temp.	37°	45°	50°	55°
Haynes . .	5·05	4·8	2·05	0·9	<0·7
Saukett . .	4·05	3·8	2·8	<0·7	<0·7

not damaged by freezing and thawing up to 4 times. It did deteriorate detectably when held at 37° for several days. The "half life" of infectivity under these conditions was less than 12 hr.

A 1/50 dilution of culture fluid containing Haynes virus was made in buffers, acids, and alkalis at 37°. One hour later, to stop the reaction, a 1/50 dilution of these was made in 10 per cent horse serum in 0·1 M phosphate buffer pH 7, and these dilutions were inoculated into monkey kidney cultures. Fig. 12 shows graphically the titre of virus found and indicates that it is stable from pH 3–9. Similar results were obtained in other experiments including one employing mouse muscle as the source of virus. Treatment for 18 hr. at 4° with an equal volume of anaesthetic ether did not reduce the titre of virus which must therefore be classified as ether stable.

Ultracentrifugation and ultrafiltration.—Preliminary experiments indicated that the virus sedimented in a way similar to that of poliovirus. The rate of

sedimentation of virus infectivity was measured using a virus suspension in a sucrose density gradient as described by Melnick, Rhian, Warren and Breese (1951). The Spinco S40 head rotating at 20,000 r.p.m. for 60 min. was used. Twelve successive layers of fluid 5 mm. deep were removed from the centrifuge tube by a needle and syringe and each layer was titrated once or twice in monkey

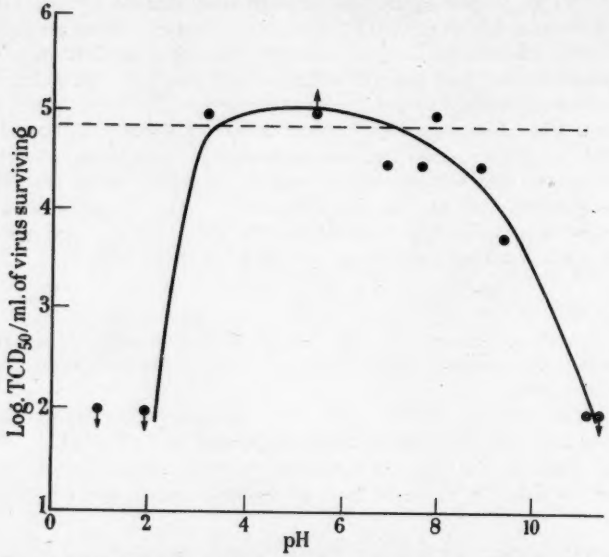


FIG. 12.—The effect of holding virus (Haynes) at 37° for 1 hr. in fluids at various pH. The titre of the untreated control is shown by the broken line.

kidney cultures. The results are shown in Table IV and the rapid rise in virus concentration between sample 3 and 5 was taken to indicate that the boundary lay between them at sample 4. From the position of the boundary and the measurements of the rotor (Spinco S40) it was possible to calculate a sedimentation constant of 179 Svedberg units assuming the viscosity of the sucrose was the same as that of water. Assuming the particles to have a density of 1·3 and a spherical shape, one may calculate the diameter of 33 mμ.

TABLE IV.—*Ultracentrifugation of Virus (Strain Haynes) in Inclined Tubes in a Sucrose Density Gradient*

	Titre of virus on sample number*											
	1	2	3	4	6	6	7	8	9	10	11	12
Titration set I .	2·33	2·18	2·17	3·25	3·83	—	—	3·83	—	—	4·0	5·0
Per cent of original titre	0·5	0·3	0·3	4·0	15	—	—	15	—	—	22	220
Titration set II .	3·16	—	<3	—	4·11	—	4·0	—	4·16	—	—	—
Per cent of original titre	3	—	<2	—	28	—	22	—	31	—	—	—

* Sample number 1 was obtained from the top of the tube and sample number 12 from the bottom.

Samples of tissue culture fluids were filtered through Gradocol membranes (supplied by the Wright-Fleming Institute, St. Mary's Hospital, London) previously " satisfied " with 10 per cent horse serum, and the titre of the filtrates was measured in monkey kidney cultures. The results of experiments of this sort are shown graphically in Fig. 13 which indicates that some of the virus passed a membrane of apparent pore diameter 58 mμ. This result is similar to that obtained with Coxsackie and polio viruses (Melnick *et al.*, 1951; Sabin, Hennessen and

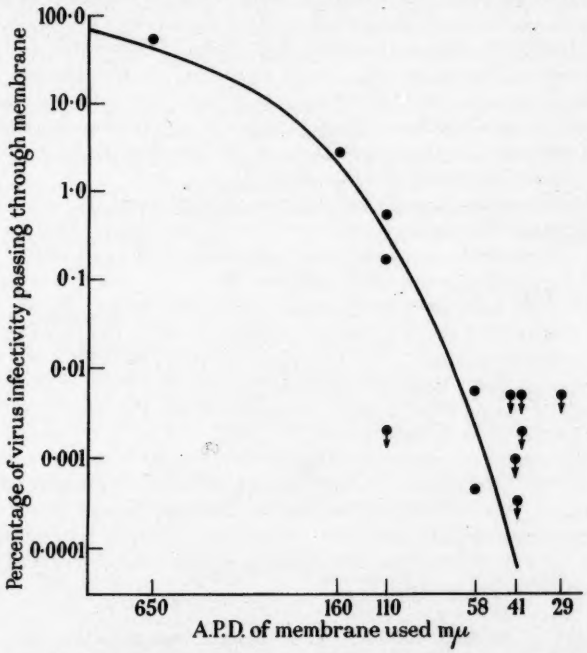


FIG. 13.—The results of experiments on filtration of pools of virus (Haynes) through collodion membranes. The points with arrows denote experiments in which no virus was detected in the filtrate.

Warren, 1954). If the figure is corrected according to the data of Markham, Smith and Lea (1942) these results indicate a particle diameter of 47 mμ. If corrected according to the data of Elford the diameter is 20–29 mμ.

Serological Studies

The neutralization test based on that used for polio-viruses gave very satisfactory results. The end-points were reproducible but when incubation was prolonged they appeared to shift more rapidly than those found with poliovirus. Sera from immunized mice, rabbits and monkeys all contained specific neutralizing antibody in titres ranging from 50–8000. Viruses obtained from patients were typed by mixing with 1/20 dilution of acute and convalescent phase serum

from an infected patient and normal and immune animal serum. Clear-cut neutralization was obtained in all cases except with mouse serum which had a low titre and gave irregular results. It was only used in preliminary experiments.

Satisfactory complement-fixing antigens were made by 3 methods. Firstly the supernatants from high-titre infected monkey kidney cultures in bottles were clarified by low-speed centrifugation and heated at 56° for 30 min. Secondly one strain (825) which seemed highly pathogenic for suckling mice was passed twice in these animals and on the third pass a 20 per cent suspension of muscle and bone was made and purified as described elsewhere (Tyrrell, Balducci, Snell and Zaiman, 1957). Both these antigens had titres of between 1 and 4 and showed increasing levels of antibody when used to titrate paired human sera and sera from immunized animals. The third method was to concentrate virus from tissue cultures in the ultracentrifuge. These antigens were anticomplementary at times. Examples of the results obtained in cases of meningitis have been published elsewhere (Tyrrell, Lane and Snell, 1958).

The results of neutralization tests and of complement-fixation tests using the antigen prepared in mice were obtained with sera from 88 control and miscellaneous subjects. In 9 sera both tests were positive and in 59 both tests were negative. In 10 the neutralization test was positive while the complement-fixation test was negative ; this may have been due to the lower sensitivity of the latter test as is seen in the results with sera from epidemic cases (Tyrrell, Lane and Snell, 1958). In 10 the complement-fixation test was positive while the neutralization test was negative. This result suggested that, as in animals, antigenic stimulation by a related virus may produce antibodies detectable by the complement fixation test but not by the neutralization test.

Neutralization and complement-fixation tests were done on certain sera which had been collected in the past 4 years from normal subjects and cases of various infectious diseases (the miscellaneous group). The age of each donor was available. The overall results have already been presented (Tyrrell, Lane and Snell, 1958). There was no obvious connection between the age of the subject and the frequency of a positive neutralization test.

The relationship of the virus to other Coxsackie A viruses

Complement fixation tests were carried out using a mouse tissue antigen and immune sera prepared in mice against Coxsackie A viruses types 1–10. Fixation was found only with Coxsackie A9 serum. The experiment shown in Table V was then carried out and the results indicated that there was a significant antigenic

TABLE V.—*The Antigenic Relationship to Coxsackie A9 Virus as Shown by Complement Fixation Tests*

Antigen used		Titre obtained using serum from mice immunized with	
Strain	Method of preparation	Coxsackie A9	Sheffield (Haynes strain)
Cambridge, Bourn	Heated tissue culture fluid	10	80
Sheffield, 825	Mouse muscle extract	12	32
Coxsackie A9	Ultracentrifuged mouse muscle extract	112	4
,, ,,	Mouse muscle extract	64	8

relationship between the two viruses, such as is found between some other members of the Coxsackie A group (Dalldorf and Sickles, 1956). Cross neutralization tests were carried out using the above sera, a Coxsackie A9 antiserum prepared in hamsters and a rabbit antiserum prepared against strain Haynes. The Coxsackie A9 sera appeared to be specific but the rabbit serum which neutralized Haynes to a titre of about 256 neutralized Coxsackie A9 at a dilution of 1/20 in some experiments. Coxsackie A9 virus (50 TCD_{50}) was not neutralized by rabbit serum against ECHO 9 or monkey serum against Sheffield 864. Eleven strains were not neutralized by a potent hamster serum against Coxsackie A9. Eighteen pairs of human sera which fixed complement with epidemic "Coxsackie ECHO 9" viruses were also titrated with Coxsackie A9 antigen. Thirteen were positive with this antigen and the titres correlated roughly with those found with "Coxsackie ECHO 9" antigen. Of 9 pairs of sera which showed rising titres with the "Coxsackie ECHO 9" viruses, 3 also showed rises with Coxsackie A9 antigen. However 5 pairs which showed marked rises in antibody titre in neutralization tests with "Coxsackie ECHO 9" viruses showed no rise and in 3 cases, no antibody, when tested with Coxsackie A9 in neutralization tests. These results confirmed the results of tests with animal sera and indicated that there is some antigenic relationship between the two viruses but that they are quite distinct.

Comparison of strains from different outbreaks

Through the kindness of a number of workers we have been able to compare the virus strains which they have sent to us as representative of the many they have recovered in the various epidemics of aseptic meningitis and exanthem which they have studied. The chief relevant data are summarized in Table VI.

TABLE VI.—*Comparison by Neutralization Test of the Antigenic Properties of 9 Strains from Various Countries*

Virus	Year isolated	Place isolated	Titre using serum prepared* against					
			Haynes	Q.E.	Ipswich	Sas. 3	1043	Echo 9
Haynes	1956	Sheffield	*250*	4,000	4,000	500	4,000	800
Q.E.	1954	London, England	1,000	*4,000*	2,000	250	4,000	1,600
Ipswich	1956	Ipswich, England	250	8,000	*2,000*	500	4,000	800
Pi 3	1955	Italy	250	—†	—	*500*	4,000	800
1043	1956	Germany	250	2,000	—	1,000	*6,000*	800
Echo 9, Hill	1953	Cincinnati, Ohio	500	2,500	—	1,000	6,000	*12,000*
Bourn	1956	Bourn, England	250	3,000	—	250	4,000	800
Dodds	1956	Huntingdon, England	250	1,000	2,000	250	8,000	200
Clegg	1956	Toronto, Canada	500	2,000	8,000	125	16,000	800

* In rabbits or monkeys.
† — not done.

This shows that although the viruses were isolated over the period from 1954 to 1956, and from places many thousands of miles apart, they were serologically indistinguishable by neutralization tests. (We find a variation of up to fourfold on retesting immune sera by the procedure used.) The epidemic viruses may have been slightly different from the prototype ECHO 9 strain (Hill) isolated from a normal child (Committee on ECHO Viruses, 1956). By contrast there seemed to be a considerable variation in the pathogenicity of these viruses for suckling mice. All strains produced microscopic lesions like those described above when tissue

TABLE VII.—*Relative Pathogenicity of Strains for Tissue Cultures and Suckling Mice*

Strain of virus	Number of tissue culture passages	Number of mouse passages	Results of titration of a virus pool: In monkey kidney cultures log. TCD_{50}/ml.	In suckling mice log. PD_{50}/ml.†	Ratio log. $\frac{TCD_{50}}{PD_{50}}$
Echo 9	13	None	5·05	<1·5	>3·55
Dodds	3	,,	6·55	<1·5	>5·0
Sheffield, Haynes	11	,,	6·0	3·0	3·0
Bourn	4	,,	7·05	5·0*	2·05
Sheffield, 825	1	,,	7·55	3·9	3·65
,, ,,	1	3	7·05	6·0	1·05
Coxsackie A9	*Nil*	24	6·8	6·3	0·5

* Undiluted virus produced no disease in suckling mice but virus diluted 10^{-1} did do so.
† PD_{50} = Fifty per cent paralytic dose.

culture material was inoculated into suckling mice. Table VII summarizes experiments on those epidemic strains which had been given few tissue culture passages and in which the details of these passes were fully known. These results showed a variation in the ratio of TCD_{50} titre to paralytic dose (PD_{50}) for suckling mice which was outside the range of experimental error, and in fact covered completely the range between the ratios for the ECHO 9 and Coxsackie A9 prototype viruses.

DISCUSSION

We have discussed elsewhere the evidence that infection with this virus causes illness in man with invasion of the central nervous system in some cases. Here we wish to emphasize that although this virus was not recovered from faeces by mouse inoculation, yet on passage to suckling mice it regularly produced symptoms and microscopical changes exactly like those of accepted Coxsackie A viruses, and after a few passages in mice gave the same ratio of infectivity in mice and tissue culture as did a mouse-passaged line of Coxsackie A9 virus. After reporting it as a Coxsackie A virus (Tyrrell and Snell, 1956) we readily confirmed the finding of other workers that it is serologically related to ECHO virus type 9. The present paper suggests that it may be serologically slightly different from the prototype ECHO 9 and that it shares some antigens with Coxsackie A9 virus. Krech (1957) has also reported that it is antigenically related to *Herpes simplex* virus.

In physical properties our virus is as stable to heat, pH and ether treatment as polio, ECHO and Coxsackie viruses. It is on the upper side of the range of sizes usually given for polioviruses and Coxsackie viruses. In addition the morphological changes produced in infected monkey kidney cells in tissue culture are identical with those shown by polio, Coxsackie and ECHO viruses (Barski, 1957). However in large doses it produces no damage to nerve cells in the monkey C.N.S.

Table VIII indicates roughly the results obtained in various tests intended to classify the virus. The Table also shows the results usually found when such tests are applied to " typical " strains of poliovirus and other viruses. It is clear that although these viruses may be subdivided a great deal by serological typing they are usually brought together into three main groups by two pathogenicity tests ; first, examination of the C.N.S. of infected monkeys for degeneration of

TABLE VIII.—*Usual Results of Tests on Certain Viruses Found in Human Faeces*

Virus	Resistance to heat, ether and high and low pH.	A.P.D. of finest collodion membrane passed mμ	Pathogenicity for	
			Suckling mice	Monkey C.N.S.
Poliovirus	Resistant	39–60†	−	+
Coxsackie virus A and B	„	32–50	+	−
Sheffield strains (described in this paper)	„	58	+	−
ECHO viruses	„ *	38*	−	−

† Sabin, Hennessen and Warren (1954).
* Melnick (1955).

anterior horn cells, and secondly, tests of the ability of the virus to cause disease and microscopical changes in suckling mice. This has been a very useful method of subdivision but it seems to be an unsound method of classification in the present state of knowledge. The difficulties which arise include the following. Poliovirus type 2 may be adapted to suckling mice (Casals, Olitsky and Anslow, 1951) and can cause lesions in the muscle and fat pads of cortisone-treated hamsters which resemble those produced by Coxsackie viruses (see Aronson and Schwartzman, 1953, 1956). Laboratory manipulation or natural selection may lead to strains of virus neutralized by antipoliovirus sera but only slightly pathogenic for the monkey C.N.S. (*e.g.*, Sabin, 1956). Coxsackie type A viruses may produce lesions of the mouse and monkey C.N.S. like those produced by polioviruses (Dalldorf, 1957, Habel and Loomis, 1957). Strains of virus exist in which some isolates may be pathogenic for suckling mice while others may not—" Coxsackie ECHO 9 " virus being an example. Finally the ability to invade the human C.N.S. is found in Coxsackie B viruses and in some types of both Coxsackie A and ECHO viruses. The ability to multiply in tissue cultures of different sorts has so far given no clear-cut confirmation of the present subdivision of these viruses. On the other hand all these viruses are found in the human alimentary tract and are of about the same size and stability; many of them cause a particular sort of degeneration in monkey kidney cells.

For practical reasons it may be necessary to continue to use the present system for identifying viruses but from the biological point of view it seems best to regard them as one family to be called perhaps " the enteroviruses " (after the pattern of the adenoviruses). The individual members would be as varied as the streptococci but in regarding them as one group our minds would be open to the similarities we can expect to find between various members in their ability to infect man and animals and in their antigenic constitution. Such ideas must have been in the minds of many workers in this field, and were indeed implicit in the title of a recent paper (Hsiung and Melnick, 1957), but it seemed worthwhile to formulate them definitely at this time as we were reporting results which show clearly the difficulties of the present method of classification.

SUMMARY

Thirty-three strains of virus were isolated from patients in an epidemic of meningitis and rash, by inoculation of throat swabs, faeces or C.S.F. into tissue cultures of monkey or human kidney cells.

The strains caused paralysis and death in suckling mice with degenerative changes in striated muscle, and multiplied in human or monkey cells in tissue culture producing cell degeneration. It produced slight fever in monkeys but no changes in the C.N.S.

The heat stability of the virus resembled that of poliovirus and it was not inactivated by ether treatment. It was destroyed rapidly in solutions with a pH less than 3 and greater than 9.

Ultracentrifugation suggested a sedimentation constant of 179 S (equivalent to a particle diameter of 33 mμ) and in ultrafiltration experiments the virus passed a membrane of 58 mμ A.P.D.

Strains isolated in similar epidemics from other parts of the world were identical when tested by cross neutralization tests. There appeared to be an antigenic relationship to Coxsackie A virus type 9, as shown by complement fixation tests on animal and human sera.

It is suggested that the polioviruses, Coxsackie viruses and ECHO viruses form one group of viruses.

We wish to thank Dr. G. Dalldorf for supplying the type strains of Coxsackie viruses and Dr. C. R. Coid for inoculating 4 monkeys. We wish to thank Mr. P. Ramsbottom and Miss N. Rogers for technical assistance. We are indebted to Professor C. H. Stuart-Harris for reviewing the manuscript. This work was done with financial assistance from the National Fund for Poliomyelitis Research.

Since submitting this article we have learned that the Committee on ECHO viruses has extended its work to include the classification of Coxsackie and polioviruses and has altered its name to the Committee on Enteroviruses, National Foundation for Infantile Paralysis. The reasons given for adopting this new name to describe the three groups of viruses are very similar to those put forward in this paper and are given in a paper by the committee (*Amer. Jl Pub. Health*, (1957), **47**, 1566).

REFERENCES

ARONSON, S. M. AND SCHWARTZMAN, G.—(1953) *Arch. Path.*, **56**, 557.—(1956) *Amer. J. Path.*, **32**, 315.

BALDUCCI, D., ZAIMAN, E. AND TYRRELL, D. A. J.—(1956) *Brit. J. exp. Path.*, **37**, 205.

BARSKI, G.—(1957) *Ann. Inst. Pasteur*, **93**, 142.

BEALE, A. J., STEVENS, P. F., DAVIS, N., STACKIW, W. AND RHODES, A. J.—(1956) *Canad. J. Microbiol.*, **2**, 298.

BOISSARD, G. P. B., STOKES, L. J., MACRAE, A. D. AND MACCALLUM, F. O.—(1957) *Lancet*, i, 500.

BUCKLEY, S. M.—(1956) *Arch. ges. Virusforsch.*, **6**, 388.

CASALS, J., OLITSKY, P. K. AND ANSLOW, R.—(1951) *J. exp. Med.*, **94**, 111.

CHANG, R. S-M.—(1954) *Proc. Soc. exp. Biol., N.Y.*, **87**, 440.

COMMITTEE ON ECHO VIRUSES.—(1956) *Science*, **122**, 1187.

CRAWFORD, M., MACRAE, A. D., O'REILLY, J. N.—(1956) *Arch. Dis. Childh.*, **31**, 182.

DALLDORF, G.—(1957) *J. exp. Med.*, **106**, 69.

Idem AND SICKLES, G. M.—(1956) In ' Diagnostic Procedures for Virus and Rickettsial Diseases '. Ed. T. Francis Jr. New York (American Public Health Association).

GIFFORD, R. AND DALLDORF, G.—(1951) *Amer. J. Path.*, **27**, 1047.

HABEL, K. AND LOOMIS, L. N.—(1957) *Proc. Soc. exp. Biol., N.Y.*, **95**, 597.

HARDING, C. V., HARDING, D., MCLIMANS, W. F. AND RAKE, G.—(1956) *Virology*, **2**, 109.
HSIUNG AND MELNICK, J. L.—(1957) *J. Immunol.*, **78**, 129.
KRECH, U.—(1957) *Virology*, **4**, 185.
LAFOREST, R. A., MCNAUGHTON, G. A., BEALE, A. J., CLARKE, M., DAVIS, N., SULTANIAN, I. AND RHODES, A. J.—(1957) *Canad. med. Ass. J.*, **77**, 1.
LENNARTZ, H., MAASS, G. AND KERSTING, G.—(1957) *Klin. Wschr.*, **35**, 327.
MARKHAM, R., SMITH, K. M. AND LEA, D.—(1942) *Parasitology*, **34**, 315.
MCLEAN, D. M. AND MELNICK, J. L.—(1957) *Proc. Soc. exp. Biol., N.Y.*, **94**, 656.
MELNICK, J. L.—(1955) *Ann. N.Y. Acad. Sci.*, **61**, 754.
Idem, RHIAN, M., WARREN, J. AND BREESE, S. S.—(1951) *J. Immunol.*, **67**, 151.
NEVA, F. A. AND ENDERS, J. F.—(1954) *Ibid.*, **72**, 307.
QUERSIN-THIRY, L., NIHOUL, E. AND DEKKING, F.—(1957) *Science*, **125**, 744.
RAMOS-ALVAREZ, M. AND SABIN, A. B.—(1954) *Proc. Soc. exp. Biol., N.Y.*, **87**, 655.
REISSIG, M., HOWES, D. W. AND MELNICK, J. L.—(1956) *J. exp. Med.*, **104**, 289.
REPORT FROM MEDICAL RESEARCH COUNCIL BIOLOGICAL STANDARDS CONTROL LABORATORY.—(1957) *Brit. med. J.*, **2**, 124.
RITA, G., RUSSI, M. AND FISCHER-FANTUZZI, L.—(1956) *Riv. Ist. sieroter. Ital.*, **31**, 286.
ROBBINS, F. C., WELLER, T. H. AND ENDERS, J. F.—(1952) *J. Immunol.*, **69**, 673.
SABIN, A. B.—(1956) *J. Amer. med. Ass.*, **162**, 1589.
Idem, HENNESSEN, W. A. AND WARREN, J.—(1954) *Proc. Soc. exp. Biol., N.Y.*, **85**, 359.
SCHERER, W. F., SYVERTON, J. T. AND GEY, G. O.—(1953) *J. exp. Med.*, **97**, 695.
TYRRELL, D. A. J., BALDUCCI, D., SNELL, B. AND ZAIMAN, T. E.—(1957) *J. Hyg., Camb.*, in press.
Idem AND CAMERON, A. H.—(1957) *J. Path. Bact.*, **74**, 37.
Idem KEEBLE, S. A. AND WOOD, W.—(1956) *Brit. med. J.*, **1**, 598.
Idem, LANE, R. AND SNELL, B.—(1958) *Quart. J. Med.*, in press.
Idem AND SNELL, B.—(1956) *Lancet*, ii, 1028.
WELLER, T. H., ROBBINS, F. C., STODDARD, M. B. AND FLORENTINO, G. L.—(1953) *J. Immunol.*, **71**, 92.
ZITCER, E. M., FOGH, J. AND DUNNEBACKE, T. H.—(1955) *Science*, **122**, 30.

THE EFFECTS OF NITROGEN MUSTARD (METHYL BIS (β-CHLOROETHYL) AMINE HCl) ON THE IMMUNOLOGICAL RESPONSE OF THE RABBIT. I. THE EFFECTS OF NITROGEN MUSTARD ON THE PRIMARY RESPONSE TO A BACTERIAL ANTIGEN

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SINCE Gilman and Philips (1946) reviewed the effects of the nitrogen mustard group of substances on the tissues of animals, various authors (Philips *et al.*, 1947; Spurr, 1947; Bukantz *et al.*, 1949; Schwab *et al.*, 1950; Marshall and White, 1950) have described the effects of nitrogen mustard on the production of antibodies, using as antigens either soluble proteins or toxins or large or multiple injections of bacterial suspensions by the subcutaneous, intramuscular or intravenous routes. They showed that under these various experimental conditions, there is a delay in the appearance of antibodies; that titres tended to be lower when antigens were injected following doses of nitrogen mustard; that when the antigen was injected 2–3 weeks before the nitrogen mustard there was no increase in circulating antibodies following the injection of the nitrogen mustard.

The present study was undertaken to observe the effect of a standard course of nitrogen mustard on the production of antibody stimulated by one dose of a bacterial suspension (100 million *Salm. typhi* H cells) injected intravenously at varying times with respect to the nitrogen mustard course. Total and differential leucocyte counts were performed to see whether they might have any relation to the production of antibody.

MATERIALS AND METHODS

Salm. typhi suspension.—This was prepared from a laboratory strain which was rendered highly motile by the method of culture in semisolid agar in a double tube. The organisms were harvested in 0·5 per cent formol saline and stored at 4° in a concentration of 2000 million organisms/ml. using Brown's Scale. The suspension was diluted to give 100 million organisms/ml. for intravenous injection.

Nitrogen mustard solution.—Methyl bis (β-chloroethyl) amine HCl (Mustine-Boots), hereafter called nitrogen mustard, was used in the experiments. Since this compound is unstable after 30 min. when dissolved in normal saline, the solution was made up and the animals injected without delay. The solution contained 0·5 mg./ml. nitrogen mustard and the injection was made into the marginal ear vein of the rabbits using a dosage of 1 mg./kg. body weight. The rabbits used weighed 2 kg. approximately.

Schedule of dosage of nitrogen mustard.—The schedule of dosage used was modified from that employed by Schwab *et al.* (1950). A course of four doses of 1 mg. nitrogen mustard was injected at daily intervals in six groups of rabbits, each group containing 3 animals. One group of 3 animals received no nitrogen mustard.

The injection of *Salm. typhi* suspension varied from 72 hr. before the beginning of the course to 24 hr. after the end of the course.

Table I shows the schedules used.

TABLE I.—*Schedule of Dosage of Nitrogen Mustard Used in Experiment and Times of Injecting Antigen*

Animal group	Time in days nitrogen mustard course							
I	A	—	—	—	—	—	—	—
II	A	—	—	D	D	D	D	—
III	—	A	—	D	D	D	D	—
IV	—	—	A	D	D	D	D	—
V	—	—	—	D A	D	D	D	—
VI	—	—	—	D	D	A D	D	—
VII	—	—	—	D	D	D	D	A

A = 100 million *Salm. typhi* H intravenously.
D = 1 mg. methyl bis (β-chloroethyl) amine HCl/kg. body weight.

Agglutination tests.—Blood was drawn off from the marginal ear vein, the serum separated and doubling dilutions made from 1/12·5 upwards in 0·25 ml. amounts and 0·25 ml. of 2000 million *Salm. typhi* H suspension added to each dilution. The tubes were incubated for 2 hr. at 56° and read with × 10·5 magnification. Agglutination tests were made at intervals over a period of 30 days from the time of injection of the antigen; at daily intervals for 14 days following the injection of antigen and thereafter every 48 hr. or 72 hr.

Leucocyte counts.—Total leucocyte and differential white counts were carried out at the same time as the blood was withdrawn for agglutination tests.

RESULTS

Relation of Time of Injection of Antigen to Nitrogen Mustard Course on Antibody Production

Table II shows the total white cell counts and times of appearance of antibody and attainment of maximum titre as well as indicating the treatment of the various groups of animals.

It will be seen that the types of response obtained can be divided into two main categories:

(*a*) Groups in which the antigen was injected before the nitrogen mustard.

(*b*) Groups in which the antigen was injected at the beginning of the nitrogen mustard course or later during the course or after the course.

Groups in which antigen was injected at least 24 hr. before the beginning of the nitrogen mustard course (Groups II, III and IV)

It will be seen from Table II that the animals in Groups II, III and IV showed no delay or only 24 hr. delay in the appearance of antibody compared with the controls (Group I); the total leucocyte counts were normal at the time of injection of antigen and rose in the following 24 hr.; there was a long delay between the

TABLE II.—*Treatment of Animals in Groups I–VII, Total Leucocyte Counts at Time of Injection of Antigen, 24 hr. Later and at Times of Appearance of Antibody and Attainment of Maximum Titre*

Titres are also shown at time of appearance and at maximum titre.

	Total leucocyte counts in 100's/c. mm. at				Agglutination titres	
	Time of injection of antigen	24 hr. after antigen	Appearance of antibody	Time of maximum titre	Time of appearance*	Time of maximum titre*
Group I—						
Antigen only given . .	10·4	12·2	10·0	9·3	3/25	5/1600
	9·8	11·4	8·8	9·2	3/25	5/1600
	11·2	12·2	10·7	11·2	3/25	5/1600
Group II—						
Antigen given 72 hr. before N.M. course	10·8	14·1	10·2	2·3	3/25	5/1600
	9·6	15·6	11·2	4·2	3/100	5/800
	10·6	12·6	8·6	2·8	3/50	5/800
Group III—						
Antigen given 48 hr. before N.M. course	9·8	14·4	6·6	10·8	3/25	21/3200
	8·4	10·8	5·8	10·0	3/25	21/800
	10·4	12·8	7·2	9·5	3/25	19/800
Group IV—						
Antigen given 24 hr. before N.M. course	10·4	20·4	1·2	11·0	4/25	17/3200
	10·8	13·0	1·7	9·8	4/25	15/1600
	9·6	12·4	1·1	10·2	4/25	17/1600
Group V—						
Antigen given at beginning of N.M. course	10·4	7·7	4·3	11·2	7/25	16/1600
	10·6	9·8	3·1	9·2	6/25	12/800
	9·7	8·2	2·7	10·2	6/25	14/800
Group VI—						
Antigen given midway through N.M. course	5·0	1·5	4·8	6·1	5/25	8/800
	4·2	2·2	4·2	7·3	6/25	9/200
	6·1	3·2	6·3	10·6	7/25	10/200
Group VII—						
Antigen given 24 hr. after N.M. course	1·4	1·2	10·4	9·6	8/25	11/1600
	1·8	1·6	10·7	12·0	8/25	11/1600
	1·3	1·0	9·6	9·5	8/25	11/800

* Numerator = day following antigen. Denominator = reciprocal of titre on that day; *e.g.*, 3/25 = third day after antigen, titre = 1/25.

appearance of antibody and the attainment of maximum titre in Groups III and IV.

In Groups II, III and IV antibody production has begun before the effect of the nitrogen mustard has occurred (using as an indication of the effect of nitrogen mustard the behaviour of the circulating leucocytes) and having begun appears to continue even when the effects of the nitrogen mustard are manifest, notably in Group II. Maximum titre is not attained in Groups III and IV until the animal has recovered, the white cell counts having returned to normal in the meantime.

Groups in which antigen was injected at the beginning of the nitrogen mustard course, or midway during the course, or 24 hr. after the course

The animals of Groups V, VI and VII show a delay of 2–3 days at least in the appearance of antibodies, actually 5 days in the case of Group VII compared with the controls (Group I); the total leucocyte counts are either normal (Group V) or depressed (Groups VI and VII) at the time of injecting antigen and they did not rise 24 hr. later; there is less delay in the attainment of maximum titre after the appearance of antibody than in the case of Groups III and IV. These points are shown forth in Table II.

Further it will be seen that there is a gradation of response from Groups III to VII typified by a lengthening of the time interval between the injection of antigen and the appearance of antibody and a shortening of the interval between the appearance of antibody and the attainment of maximum titre.

Relation of Leucocyte Counts to Antibody Production

In all the animals which received a course of nitrogen mustard the total leucocyte counts fell to a minimum 48 hr. or 96 hr. after the end of the course and recovered in the following 7 days or so.

It has been noted that there is either no delay or a minimal delay in the appearance of antibody in those animals in which the total leucocyte counts were normal at the time of injection of antigen and for at least 24 hr. later (Groups II, III and IV) and a maximal delay in those animals in which the total leucocyte counts were lowest at the time of injection of antigen (Group VII). It is not suggested that there is a direct causal relationship between the behaviour of the total leucocyte counts and the production of antibody, probably both are manifestations of the toxic effects of the nitrogen mustard without their being causally connected but the possibility exists that there is a connection between the behaviour of the polymorphonuclear leucocyte count and the production of antibody. The polymorphonuclears are well established phagocytes and Sabin (1939) and Smith (1956) among many authors have shown that these cells carry soluble proteins and bacteria.

Since the administration of nitrogen mustard causes a granulo-cytopenia of a severe degree, the virtual absence of a system of phagocytes may well in part account for the delay in appearance of antibody since there may be a delay in the phagocytosis and therefore the breakdown of antigen.

Table III shows the correlation between the time of injection of antigen, the polymorphonuclear counts and the time of appearance of antibody and attainment of maximum titre.

The following points may be seen from Table III.—In the case of Groups II, III and IV the polymorphonuclear counts were normal at the time of injection of the antigen and for at least 24 hr. later and there is either no delay or only a minimal delay in the appearance of antibody. It is suggested that in these groups of animals breakdown of the antigen has occurred with little or no delay with a resultant normal time of appearance of the antibody, the long delay in Groups III and IV before the attainment of maximum titre being due either to delay in the synthesis of antibody or to delay in complete breakdown of the antigen as a result of the effects of the nitrogen mustard.

TABLE III.—*Relation between Polymorphonuclear Counts at Time of Injection of* Salm. typhi *H and 24 hr. after, also shows Time of Appearance of Antibody and of Attainment of Maximum Titre*

		Polymorphonuclear counts in 1000's/c. mm.. at		Agglutinins	
		Time of antigen	24 hr. after antigen	Time of appearance (days)	Time of max. titre (days)
Group I	Antigen only given	4·7	7·0	3	5
		4·3	6·9	3	5
		4·7	6·3	3	5
Group II	Antigen 72 hr. before N.M. course	4·4	7·1	3	5
		3·6	7·4	3	5
		4·7	6·9	3	6
Group III	Antigen 48 hr. before N.M. course	4·0	9·4	3	21
		3·7	3·5	3	21
		4·4	6·6	3	19
Group IV	Antigen 24 hr. before N.M. course	4·8	12·6	4	17
		4·4	6·5	4	15
		4·5	7·4	4	17
Group V	Antigen at beginning of N.M. course	4·8	4·0	7	16
		5·1	5·8	6	12
		4·5	4·2	6	14
Group VI	Antigen midway through N.M. course	2·6	0·2	5	8
		2·0	0·6	6	9
		2·9	0·7	7	10
Group VII	Antigen 24 hr. after N.M. course	0·1	0·1	8	11
		0·3	0·3	8	11
		0·2	0·08	8	11

In the case of Groups V, VI and VII the polymorphonuclear counts were either normal or reduced at the time of injection of antigen and fell in the following 24 hr. (except in the case of one animal of Group V) and there is a delay in the time of appearance of antibody; this delay in production of antibody would be, according to the suggestion just made, due to a delay in the breakdown of antigen as a result of the relative absence of the polymorphonuclear leucocytes, and also possibly due to an interference with the synthesis of antibody.

DISCUSSION

The results of the experiments show that the effects of nitrogen mustard on the antibody response of rabbits injected with 100 million *Salm. typhi* H intravenously depend on the time at which the antigen is injected with relation to the course of nitrogen mustard.

Spurr (1947) showed that when blood was taken at comparable times from animals injected with nitrogen mustard and normal animals, both groups having been injected with typhoid vaccine intramuscularly, the titres of the nitrogen mustard group were lower than those of the normal animals, but the recordings were taken at weekly intervals, so it is possible that a late rise in titre may have been missed by this method.

In the present study it would appear that a delay in appearance of maximal

titre is a constant feature in those animals in which antigen was injected 48 hr. or 24 hr. before the administration of nitrogen mustard but that a high titre was eventually reached. This finding prompts the question, " What is happening to the antigen and antibody production during the time at which the animal is under the effects of nitrogen mustard?"

The fact that normal or high titres can be obtained when the animals recover suggests that the antigen is not lost from the body during the time of maximal toxicity of the nitrogen mustard. The question arises as to whether the antigen is broken down during this stage or not, i.e., whether there is a pool of antigenic determinants as templates available but that they cannot be used due to the toxic effects of the nitrogen mustard on the animal.

There is no doubt that in the case of the animals injected 48 hr. or 24 hr. before the beginning of the nitrogen mustard course, antibody production had begun before the effects of the nitrogen mustard were maximal and indeed was continued during the phase of maximal injury. Marshall and White (1950) have shown that plasma cells are resistant to the effects of nitrogen mustard and X-rays and Benecerraf (1954) has shown that, under the influence of nitrogen mustard, although reticular and other cells are able to carry on phagocytosis, they are unable to divide and these findings would explain the low production of antibody obtained in the present study.

If one accepts Burnet's hypothesis (Burnet and Fenner, 1949 ; Burnet, 1956), namely, that once the antibody production mechanism has been initiated, the ability to produce antibodies is passed on to the progeny of the antibody producing cells (the cells of the plasma series), it is possible to see that as soon as the effects of the nitrogen mustard (which prevent mitosis occurring *inter alia*) have worn off, much more antibody will be produced by the progeny of the dividing antibody producing cells.

As regards the possibility of the slowing of the breakdown of antigen Schwab *et al.* (1950) have shown that there is a delay in the disappearance of bovine gamma globulin from the circulation of rabbits treated with nitrogen mustard or X-rays. Whether this delay is due to an effect on the R.E. cells is not known although Benecerraf *et al.* (1954) have shown that there is some effect on the phagocytic power of R.E. cells of nitrogen mustard treated rabbits when the cells have been blocked previously with carbon particles—he ascribes the loss of phagocytosis to the inability of the cells to divide.

It is possible however that the removal of the polymorphonuclear cells from the circulation by the action of nitrogen mustard may result in a slowing down of phagocytosis of the antigenic material.

When one considers what happens when the nitrogen mustard course precedes the injection of antigen, it is clear that there is a delay of several days before antibody appears and that antibody appears after the animal has begun to recover from the effects of the nitrogen mustard. In these animals there is not the long delay between appearance of antibody and attainment of maximal titre. There appear to be three possible reasons for this observed fact :

(1) That there is delay in the breakdown of antigen due to nitrogen mustard interfering with metabolic activity ;

(2) that there is delay in the synthesis of antibody following the breakdown of antigen ;

(3) that there is a combination of (1) and (2).

It is not possible to state which of the mechanisms is active although further experiments to be reported show that there appears to be a delay in the breakdown of antigen—the method used being one of the transfer of splenic tissue from donor animals injected with nitrogen mustard and antigen and later challenge of the recipients with the antigen.

SUMMARY

The effects of nitrogen mustard on the antibody response of rabbits injected with 100 million *Salm. typhi* H are related to the time at which the antigen is injected with respect to the course of nitrogen mustard.

The longest delay in appearance of antibody occurs when the antigen is injected following the nitrogen mustard; there is no delay when the antigen is injected at least 48 hr. before the beginning of the nitrogen mustard course.

In most cases maximum titre is attained when the animals have recovered from the effects of the nitrogen mustard.

The possible role of the polymorphonuclear leucocytes in the immune mechanism is discussed.

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REFERENCES

Benecerraf, B., Halpern, B. N., Biozzi, G. and Benos, S. A.—(1954) *Brit. J. exp. Path.*, **35**, 97.

Bukantz, S. C., Dammin, K. S., Johnson, M. C. and Alexander, H. L.—(1949) *Proc. Soc. exp. Biol., N.Y.*, **72**, 21.

Burnet, F. M. and Fenner, F.—(1949) 'The Production of Antibodies'. Monograph of Walter and Eliz. Hall Inst., Melbourne.

Burnet, Sir MacFarlane.—(1956) In 'Enzyme, Antigen and Virus'. Cambridge University Press.

Gilman, A. and Philips, F. S.—(1946) *Science*, **103**, 409.

Marshall, A. H. E. and White, R. G.—(1950) *Brit. J. exp. Path.*, **31**, 157.

Philips, F. S., Hopkins, F. H. and Freeman, M. L.—(1947) *J. Immunol.*, **55**, 289.

Sabin, F. R.—(1939) *J. exp. Med.*, **70**, 69.

Schwab, L., Moll, F. C., Hall, T., Brean, H., Kirk, M., Hawn, C. Z. and Janeway, C. A.—(1950) *Ibid.*, **91**, 505.

Smith, J. M. and Dubos, R. J.—(1956) *Ibid.*, **103**, 87.

Spurr, C. L.—(1947) *Proc. Soc. exp. Biol., N.Y.*, **64**, 259.

AN ATTENUATED VARIANT OF *STAPHYLOCOCCUS AUREUS* WITH SPECIFIC IMMUNISING PROPERTIES

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IMMUNITY to staphylococcal infection is as yet poorly understood. The difficulty is the lack of a satisfactory experimental method for the study of the antibacterial aspects of immunity. Intravenous infection with live staphylococci is lethal for the rabbit (Topley and Wilson, 1955) and immunisation with formalinised or boiled vaccines gives only poor protection (Cowan, 1939).

The present study arose from the chance observation, during some other work, that the culture of Cowan's serotype I staphylococcus had become less pathogenic for rabbits. This strain which had been originally isolated from a case of septic arthritis was then highly pathogenic for the rabbit (Cowan, 1939). The attenuation of this strain affords the opportunity to test its vaccine value and to study the immunological responses of the host to staphylococcal infection.

MATERIAL AND METHODS

Strains of staphylococci used.—Standard type strains of Cowan's serotype I (NCTC No. 8530) and serotype III (NCTC No. 8532) were used in experimental tests of comparative pathogenicity. A freshly isolated strain of serotype I (No. 2299) obtained from Dr. R. E. O. Williams of the Staphylococcal Reference Laboratory was also used in certain experiments.

The rabbits.—A mixed breed of rabbits, obtained from a dealer and weighing approximately 2·25 Kg. each, was used throughout the experiments.

Inocula.—In the tests of comparative pathogenicity and in challenge experiments to test the value of different methods of immunisation, the inoculum was, in every case, 0·3 ml. of an 18 hr. broth culture administered by the intravenous route.

Immunisation and challenge procedures.—Living attenuated Cowan's serotype I (NCTC No. 8530) was used as a vaccine to test its immunising potency. A single intravenous injection of 0·3 ml. of an 18 hr. broth culture was given and 20 days later the immunity was challenged with the live recently isolated serotype I (Williams, 2299).

Serotype I (Williams, 2299) was used to prepare formalinised and boiled vaccines. This strain was grown for 18 hr. at 37° on heart extract agar. The formalinised vaccine was made in 0·1 per cent formol-saline, and the other vaccine by boiling in saline at 100° for 2½ hr. The suspensions were standardised to the opacity of No. 4 of Brown's scale. Six intravenous injections of the respective antigens were given to rabbits at 3–4 day intervals beginning with 0·25 ml., increasing by 0·25 ml. till 1 ml. was reached, when 3 doses of this volume were given. One week after completion of the immunising course in each series, the immunity was challenged with live serotype I (Williams, 2299).

RESULTS

The results of the comparative pathogenicity tests are shown in Table I. The selection of strains was limited to representatives of Cowan serotypes I and III. Cowan serotype II (NCTC 8531) was not included because in a preliminary experiment it was found to kill 2 rabbits and the post-mortem appearances were

TABLE I.—*The Pathogenicity of Staphylococci for Rabbits following Intravenous Inoculation*

Serological type	Dose (ml.)	Rabbit No.	Fate of rabbit	Major post-mortem finding	Mortality ratio
Cowan I (NCTC No. 8530)	0·3	1	Paralysis.	Osteomyelitis of lumbar vertebrae	2 : 8
		2	Killed (16 days)		
		3			
		4			
		5	Alive and well (6 weeks)		
		6			
		7			
		8			
Cowan I (Williams No. 2299)	0·3	9	Died (4 days)	Multiple kidney abscesses	7 : 8
		10	„ (5 „)		
		11			
		12	„ (6 „)		
		13	„ (8 „)		
		14			
		15	„ (10 „)		
		16	Alive and well (4 weeks)		
Cowan II (NCTC No. 8532)	0·3	17	Died (2 days)	Ditto	7 : 8
		18			
		19			
		20			
		21			
		22	„ (10 „)		
		23	„ (15 „)		
		24	Alive and well (4 weeks)		

comparable to those produced by Cowan serotype III. Table I shows that Cowan serotype I (NCTC 8530) killed 2 of the 8 rabbits in the test, whereas the freshly isolated Cowan serotype I (Williams, 2299) and Cowan serotype III (NCTC 8532) caused the death of 7 out of 8 rabbits respectively. The 2 rabbits in group I (Cowan I NCTC 8530), which are shown as deaths, developed paralysis 16 days after inoculation and were killed to avoid suffering. The post-mortem findings showed osteomyelitis of the lumbar vertebrae with abscess formation. In the two other groups the major post-mortem finding was the presence of multiple abscesses in the kidneys. This experiment demonstrated the attentuation of Cowan serotype I NCTC 8530 strain for rabbits following intravenous inoculation. The possible significance of the 2 rabbits found to have osteomyelitis will be considered in the discussion.

Table II shows the results of the experiment designed to compare the protective value of different vaccines prepared from Cowan serotype I. The vaccines tested were (*a*) living attenuated NCTC 8530 strain, (*b*) formalinised freshly isolated strain (Williams, 2299) and (*c*) boiled freshly isolated strain (Williams, 2299). The methods of immunisation and challenge were described in the section entitled materials and methods. Table II illustrates the complete protection induced by the live attenuated NCTC 8530 strain, the partial protection induced by the formalinised vaccine as judged by the extension of the average time till death from 2 days for the controls to 11·5 days for the experimental group, and the failure of the boiled vaccine to induce any protection of note.

TABLE II.—*Comparison of Protection Induced by Cowan Type I Staphylococcus Vaccines in* (a) *Living Attenuated,* (b) *Formalinised and* (c) *Boiled States*

Vaccine	Challenge staphylococcus	Number of rabits: Alive and well	Number of rabits: Died	Average days till death
(*a*) Living attenuated Cowan Type I (NCTC No. 8530)	0·3 ml. Live Cowan type I (Williams, No. 2299)	5	0	0
(*b*) Formalinised Cowan Type I (Williams, No. 2299)	Ditto	0	6	11·5
(*c*) Boiled Cowan Type I (Williams, No. 2299)	,,	1	5	3·6
None given	,,	0	6	2·0

TABLE III.—*Type Specificity of Immunity Induced by Living Attenuated Cowan Type I Staphylococcus Vaccine*

Vaccine	Challenge staphylococcus	Number of rabbits: Aline and well	Number of rabbits: Died
* Living attenuated Cowan type I (NCTC No. 8530)	0·3 ml. Living Cowan type III (NCTC No. 8532)	0	4

* These are 4 of the 5 rabbits of series (*a*) of Table II, inoculated 5 weeks after experiment in Table II. The fifth rabbit was retained under observation and remained alive and well throughout the full period of the experiments.

Table III demonstrates that the protective immunity induced by the live attenuated NCTC 8530 strain of Cowan serotype I is serotype specific. In this experiment 4 of the 5 rabbits which are designated as series (*a*) in Table II and which 8 weeks previously had been inoculated with live attenuated NCTC 8530 strain and 3 weeks later with freshly isolated Cowan serotype I were now challenged with Cowan serotype III by the intravenous route. This challenge with the different serotype resulted in the death of all 4 rabbits.

DISCUSSION

In the vast literature on staphylococcal disease published since the war there have been few reports concerned with the experimental study of immunity. The intense empirical search for new antibiotics would seem to have replaced study of the mechanisms of infection and resistance in staphylococcal disease. The failure, however, of the antibiotic approach to solve the problem of hospital staphylococcal disease surely warrants a renewal of interest in the mechanisms of infection and immunity.

The present observation that Cowan's original serotype I staphylococcus (Cowan, 1939) is now attenuated for the rabbit raises interesting possibilities. This strain provides not only a useful tool for the investigation of antibacterial immunity in staphylococcal disease but also a new approach to the study of the antigenic structure of staphylococci by allowing us to prepare an antiserum using

live organisms. A further proof that the strain is of lowered virulence is shown by our finding (unpublished) that the injected organisms are more rapidly cleared from the blood, lungs, liver and spleen than Cowan's serotypes II and III, and that the only significant lesions produced are small rapidly healing abscesses in the kidneys.

The paralysis, occurring on the 16th day after intravenous injection, in 2 of the rabbits was found to be caused by osteomyelitis of the lumbar vertebrae with abscess formation. This was probably the result of minor trauma when the animal was in the " inoculation box " at the time of injection. The trauma must have been very slight, in view of the special care we took, but presumably it was enough to localise the infection, and it may be significant that Cowan (1939) reported paralysis after challenge in one of his immunised rabbits.

The complete protection induced by a single inoculation of the live attenuated strain contrasts sharply with the partial protection induced by a course of the formalinised vaccine of the virulent type I, and the lack of protection observed after boiled vaccine (Table II). The specificity of the protection is clear cut (Table II), and the complete lack of protection against Cowan's serotype III is fresh confirmation of the validity of the serological differentiation of staphylococci.

The spontaneous appearance of an attenuated variant of Cowan's serotype I with specific immunising properties justifies a search for similar variants amongst the other serotypes.

SUMMARY

Cowan's original serotype I staphylococcus is now attenuated for the rabbit Intravenous inoculation induced complete protection to challenge with a recently isolated pathogenic serotype I. Formalinised vaccine prepared from the recently isolated serotype I strain gave a partial protection ; boiled vaccine from the same strain gave none. The immunity induced by the attenuated serotype I strain was type specific as it failed to protect against challenge with serotype III. Attention is drawn to the value of the attenuated strain in the study of the mechanism of antibacterial immunity to staphylococcal infection.

We wish to thank Professors W. J. Tulloch and A. C. Lendrum for their interest in this study, Dr. S. T. Cowan for sending the type strains from the National Collection of Type Cultures, and Dr. R. E. O. Williams for giving us strain Colindale 2299.

REFERENCES

Cowan, S. T.—(1939) *J. Path. Bact.*, **48**, 545.

Topley and Wilson.—(1955) In ' Principles of Bacteriology and Immunity ' (4th Ed.). London (Arnold), p. 713.

THE ESTABLISHMENT OF STAPHYLOCOCCAL ABSCESSES IN THE MOUSE KIDNEY

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WHEN mice are infected intravenously with coagulase positive staphylococci they die with the formation of multiple renal abscesses (Gorrill, 1951). These early observations were based on the use of large inocula. The present paper is devoted to a study of the effects of injecting graded doses of staphylococci. The study has included a comparison of the death rates and the rates of renal infection since it has been found that the latter is a more sensitive indicator of infection. A number of experiments have been carried out to determine how many staphylococci are involved in the formation of a single abscess.

MATERIALS AND METHODS

Mice.—Albino mice, strain C, of both sexes, aged 8 to 10 weeks with a mean weight of 20 g., were used throughout. They were bred in a small colony at Guy's Hospital Medical School. The diet consisted of pellets (Aberdeen cube, North Eastern Agricultural Society, Aberdeen) and water *ad lib* with dried milk powder and crushed oats as supplements. Before use they were boxed at random in groups never exceeding ten.

Staphylococci.—All the main experiments were conducted with a single strain of *Staphylococcus pyogenes* No. 8354 obtained from the National Collection of Type Cultures (N.C.T.C.) Colindale, London. For certain experiments two variants of the strain were used. A streptomycin resistant mutant, 8354 S.R.; obtained by plating large numbers of staphylococcus 8354 on digest agar containing streptomycin 1000 μg./ml. This strain gave equal counts on digest and streptomycin agar. The second is a lysogenic variant, 8354/8426, prepared by infecting staphylococcus 8354 with N.C.T.C. typing phage 8426 (Gorrill and Gray, 1956). In a few experiments staphylococcus 60, a coagulase positive strain isolated from clinical material, was used.

All four strains were freeze-dried and ampoules opened when required. In general an ampoule was opened each month and a master culture set up from which each subculture was made.

Media.—In order to maintain as constant a standard as possible large batches (70–90 l.) of tryptic digest broth and agar (Southern Group Laboratory, London) were obtained. Before a new batch was accepted a sample was tested in parallel with the old.

Preparation of inoculum.—Staphylococci were grown in broth overnight and 0·1 ml. of this culture added to 9·9 ml. of broth in a simple optical tube and shaken at 37° in an apparatus previously described (Gorrill and Needs 1958). The opacity during growth was followed using a " Spekker " absorptiometer (Hilger & Watts, London). When the required opacity was reached the culture was centrifuged and resuspended in cold saline made with glass distilled water. An aliquot was taken and its opacity measured more accurately than was possible in the optical tube using a standard 1 cm. optical glass cell. Suitable dilutions were made in saline. By this method young actively growing log. phase cultures were available which as Rogers (1956) has shown have different properties from aged broth cultures.

Bacterial counts.—Broth cultures and saline suspensions were counted by making careful serial dilutions in phosphate buffer in duplicate. From suitable dilutions 0·1 ml. amounts were added to 3·0 ml. of broth containing 0·6 per cent agar at 46° in small tubes. When well mixed the contents were poured over an agar underlay, allowed to set and incubated at 37°.

Two samples were taken from each dilution so that using duplicate dilutions each level was counted 4 times. Next morning plates showing between 30 and 200 colonies were counted.

Organ counts.—Mice were killed with choloroform vapour and exsanguinated. Then under sterile precautions the organs were removed and homogenised in 10 ml. of buffer in sterile borosilicate glass tissue grinders (A. Gallenkamp & Co. Ltd., London). Serial dilutions and counts were made as for broth cultures. All counts were recorded as number of bacteria per organ or ml. of blood. When the liver was counted, owing to its size, it was found convenient to divide it into two and emulsify the two halves separately. When it was known that the organ count would be low a larger volume of emulsion, up to 1·0 ml., was added to the molten agar. In a few cases the total count was made by dividing the emulsion between ten tubes of agar and counting all the colonies that appeared.

RESULTS

Before proceeding to the detailed results two minor points merit description. As will be seen later it was important to know the number of cells in the inoculum given to the mouse. Two alternatives presented, either to keep the stock overnight at 4° while the count was made and then inject suitable dilutions or else to devise an accurate method of relating viable count to the degree of opacity. The first method gave rather unsatisfactory results so the second was developed. As described the overnight broth culture was shaken in fresh broth and an arbitrary standard opacity of 0·2 was chosen. When this figure was reached, a more accurate reading was made using an optical cell. A graph had been constructed of the viable count versus opacity for the range 0·15 to 0·25. The graph was drawn on an arithmetic scale with 95 per cent confidence limits fitted. As an indication of the degree of error, at an opacity of 0·20 the viable count was $4{\cdot}4 \times 10^7$ cells/ml. S.D.$= 0{\cdot}82 \times 10^7$.

The other point is the optimum volume of fluid in which to suspend the inoculum. In general the larger the volume administered the smaller the percentage error, but there are limiting factors. More than 1 ml. of fluid intravenously will embarrass the circulation of a mouse, while some types of broth or buffer apparently non-toxic in 0·1 ml. amounts are lethal in 1·0 ml. amounts or more. Hence 0·5 ml. was chosen as the standard volume and saline being well tolerated by the mouse was used as the suspending fluid. Since saline has the reputation of being bactericidal (Wilson, 1922), possibly due to traces of impurities, counts were made at intervals on cultures suspended in saline to see if a detectable fall occurred in the bacterial numbers before the mice were injected. This was not found to be the case.

The effect of inoculum size on mortality rate and frequency of renal infection

It had been found earlier that the majority of deaths occur within 14 days of infection. For this reason unless otherwise stated all experiments were terminated at this time and the survivors killed and examined for renal involvement. This method underestimates the true mortality rate by a small amount but gives consistent results and has been adopted by other workers (Smith and Dubos, 1956). All experiments in this section were done with staphylococcus 8354 only.

In Table I is shown the results of injecting groups of mice with decreasing numbers of staphylococci. These observations were repeated on a number of occasions with very similar results. At this point it should be mentioned that the number of abscesses in each infected kidney has not been recorded. This is due to the restriction of this paper to the problem of the incidence of infected and non-

TABLE I.—*Number of Deaths and Frequency of Renal Infection in Mice Injected with Different Doses of* Staph. aureus 8354.

Number of bacteria injected	Number of mice challenged	Number dead	Number of mice with: Both kidneys infected	One kidney infected	Neither kidney infected
$2{\cdot}1\times10^7$	21	19	21	0	0
7×10^6	24	15	20	4	0
$2{\cdot}3\times10^6$	20	8	12	5	3
8×10^5	20	4	6	6	8
$2{\cdot}7\times10^5$	20	0	1	6	13

infected kidneys. If the number of abscesses had been scored then the infected kidney group would have been subdivided.

In mice which died the cause of death was assumed to be a combination of disturbed renal function and toxaemia. The possibility that death was in fact due to a terminal breakdown of one or more abscesses with generalised multiplication was excluded by examining the blood and spleens of a number of moribund mice. The blood count was less than 100 orgs./ml. and the spleen count less than 10^4 orgs./organ.

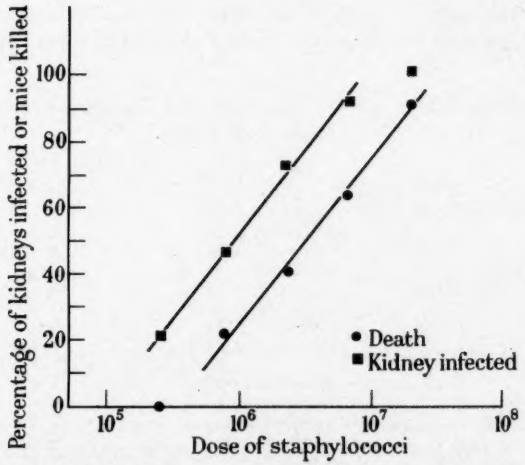

FIG. 1.—Relationship between dose and percentage of mice killed or kidneys infected.

Fig. 1 has been constructed from the data given in Table I. The lines have been drawn through the points in the 20–80 per cent range. It will be seen that the two lines are roughly parallel. In replicate experiments the values between 20 and 80 per cent were very consistent while with higher or lower values there was considerable variation.

From an inspection of the figure it is possible to estimate the number of staphylococci liable to infect any proportion of individual kidneys exposed. In particular it is possible to decide the dose infecting half, a value we may describe as the K.I.D. 50 (kidney infecting dose). Similarly it is possible to read off the probable mortality rates.

Before going further it should be said that mice surviving with renal abscesses are able to ovecome the infection and healing with scarring follows. In a separate study a large number of mice have been followed, the degree of their renal infection being calculated from Fig. 1. These mice in general appeared well despite severe renal damage. In fact a number of mice appreared to lose one kidney completely, due it is thought to involvement and destruction of the single renal papilla. Such mice have in effect undergone a form of bacteriological nephrectomy and are the object of further research. A further point that may be discussed here is the most probable distribution of infected kidneys in groups of mice. Considering the theoretical K.I.D. 50 point, here in a group of 20 mice there would be 20 infected kidneys, but what proportion of mice would possess both, one or neither kidney infected ?

A simple hypothesis was that the kidneys would be distributed according to the binomial formula, *i.e.*, $(p + q)^n$; where p = the probability a kidney would be infected, q = the probability it would not and n = the number of kidneys in a mouse (2). Hence it is possible to calculate for any value of p the distribution of infected kidneys, *e.g.*, at K.I.D. 50 $p = 0{\cdot}5$ $q = 0{\cdot}5$ $n = 2$. $(\frac{1}{2} + \frac{1}{2})^2 = 1/4$. $2/4$. $1/4$, *i.e.*, 1 in 4 mice have both kidnys infected (5 in 20), 2 in 4 one kidney infected (10 in 20) and 1 in 4 free of infection (5 in 20).

In Table II the data of Table I together with that of one other experiment are shown together with the expected values calculated from the above formula.

TABLE II.—*Application of the Binomial Distribution to the Frequency of Renal Infection*

Dose	p*	Both kidneys infected	One kidney infected	Neither kidney infected
$2{\cdot}1\times10^7$	1·0	Observed = 21	0	0
		Expected = 21	0	0
7×10^6	0·917	Observed = 20	4	0
		Expected = 20·16	3·66	0·16
$2{\cdot}3\times10^6$	0·725	Observed = 12	5	3
		Expected = 10·5	7·9	1·5
$2{\cdot}1\times10^6$	0·69	Observed = 24	14·0	7
		Expected = 21·36	19·3	4·36
			χ^2=3·3	
8×10^5	0·45	Observed = 6	6·0	8
		Expected = 4	10	6
			χ^2=3·26	
$2{\cdot}7\times10^5$	0·2	Observed = 1	6	13
		Expected = 0·8	6·4	12·8

* p = probability of a kidney being infected at the indicated dose.

Owing to the small numbers of values in some cells it is only possible to calculate. χ^2 for two examples, one being the additional experiment not shown in Table I. When calculated the value of χ^2 lies between $p = 0{\cdot}1$ (4·606), $p = 0{\cdot}2$ (3·219) and shows a good agreement between calculated and observed values. In the other results the agreement appears good although it cannot be tested.

The relationship between inoculum and kidney implant

In view of the linear relationship between the proportion of kidneys infected and the logarithm of the dose injected (over the range 20–80 per cent) it was

decided to estimate the number of bacteria deposited in the individual kidney over this dose range. Groups of 5 mice were injected with known numbers of staphylococci and killed at 30 min. The results of the enumeration of the bacteria in separate kidneys are shown in Fig. 2. The arithmetic means and 95 per cent confidence limits have been calculated and are shown. On avarage the S.D. was 30 per cent of the mean value which is small considering the errors inherent in the method. At no time was any marked difference in count between the 2 kidneys of the same mouse discovered. On the graph a line at 45° has been drawn. It will be seen that it fits the data well and so it may be argued that there is a linear relationship between inoculum and kidney implant.

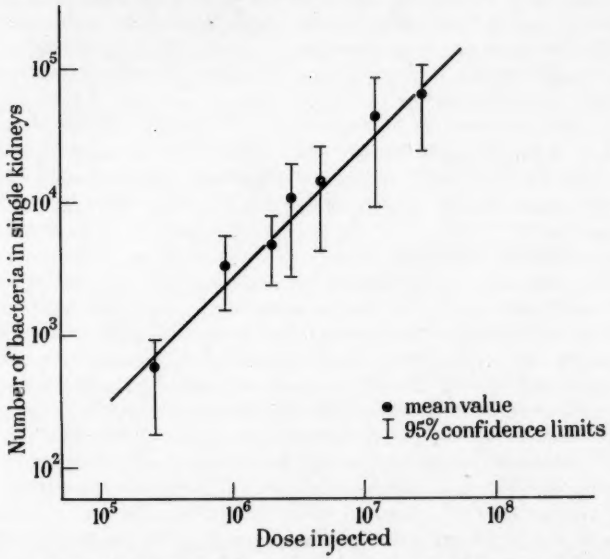

FIG. 2.—Relationship between dose and number of bacteria deposited in the kidney.

It was of interest to discover to what extent the implant at 30 min. was augmented or depleted as time went by. Groups of mice given a known dose of staphylococci were examined at intervals as described above (see Methods). It was found that the staphylococci in the reticuloendothelial system declined in numbers. The results were in fact similar to those reported by Smith and Dubos (1956). In the kidney the fate of the implant depended on the K.I.D. 50 value of the inoculum. With low values where most kidneys would escape infection the number of staphylococci declined in the majority of kidneys examined, while with high values the majority increased in number. For example, with a dose of 2 K.I.D. 50 the bacterial count had risen at 24 hr. in 13 out of 18 kidneys examined and fallen in the other 5, while with a dose of 0·5 K.I.D. 50 the count in the same time had risen in only 5 kidneys and had fallen in 15. The cause of the fall in numbers was briefly investigated by testing the ability of minced kidney to kill staphylococci *in vitro*. Using a wide range of bacterial inocula and incubating

at 37° it was not possible to demonstrate any fall in bacterial numbers although it appeared that the rate of multiplication was slowed under these conditions.

From these observations arose the conception that the abscesses found in the kidney were produced by the cells present in the implant. Hence by comparing Fig. 1 and 2 it is possible to arrive at a measure of the number of staphylococci needed to produce any proportion of infected kidneys. If this is so then it would be of interest to study the cells forming the implant more carefully to determine what factors govern their ability to produce infection.

The origin of individual abscesses in the kidney

When considering the fate of the implant it may be said that at the K.I.D. 50 dose, not only do half the kidneys suffer no ill effects, but in the infected kidneys the nunber of abscesses never approaches the number of bacteria in the implant. Hence one is faced with the problem of the need for such a large number of bacteria to produce only a few lesions.

Only two explanations will be explored in this paper: either that the implant contains a few cells that are virulent and from these are derived the abscesses or that the staphylococci are unable to multiply on their own but when in groups provide mutual support leading to rapid multiplication and abscess formation.

The effect of passage on virulence

In order to test the hypothesis of non-homogeneity of the bacterial culture the effect of serial passage on virulence was tested. Five mice were infected with 10^6 cells and on the fifth day they were killed and at autopsy well-defined isolated kidney abscesses cultured. From these cultures fresh inocula were set up and 5 more mice injected with 10^6 cells. In all, 10 serial passages were made with several lines. It was noticed that although the inoculum was constant there was no increase in abscess formation as one would expect if virulent cells were being selected. Nevertheless one tenth passage strain was titrated in groups of 10 mice against the stock 8354 and the two K.I.D. 50 values were within the normal variation of one another.

However it might be argued that the plating of the bacteria between animal infections would return the bacteria to a less virulent state. The method of serial passage using subcultures was used in order to standardise the inoculum in each group. If the kidneys were emulsified and the emulsion used as the inoculum the dose would be unknown and owing to the large amount of tissue debris it was likely that a number of non-specific deaths would follow. To overcome these difficulties a number of experiments were done as follows. Five mice were injected with 10^6 cells each, and on the fifth day they were killed and all infected kidneys removed and emulsified. The suspension was allowed to stand and the supernatant fluid passed through a clarifying filter. From this fluid serial dilutions were made and 5 mice inoculated from each and viable counts were made. The next day the number of staphylococci received by each mouse was known. Owing to the difference between the standard log phase washed cells and the cells in a heavy suspension of tissue fragments, protein etc., no very direct comparison would be feasible.

It was found that the staphylococci in the emulsion of infected kidneys appeared to be more virulent than staphylococci in saline. However there was no increase in virulence on passage. Finally saline suspensions of staphylococci added to

filtered emulsions of normal kidneys also showed an increase in virulence on inoculation. It appears therefore that while it is possible to increase the virulence of staphylococci by altering the suspending agent the staphylococci are unchanged by serial mouse passage.

The results of using mixed infection

The alternative theory was now explored, *i.e.*, that abscesses follow the chance deposition of a number of staphylococci in such close proximity that they were able mutually to support one another through the early phase of establishment. If this theory were true the majority of abscesses would be derived from more than one staphylococcus. If mice were infected with mixtures of staphylococci this would be reflected in the bacterial population of individual abscesses.

In order to set up satisfactory experiments it was essential to obtain strains of staphylococci with similar K.I.D. 50 values, equal growth rates in mice and some marker system which would allow the strains to be recognised in the presence of one another.

The early experiments relied upon the use of phage typing as a method of distinguishing between different staphylococci. Three strains were employed, 8354, 8354/8426 and 60. All had similar K.I.D. 50 values, and growth rates in mice, and were readily distinguishable by phage typing. The experiments were conducted as follows: each strain was grown separately and diluted so that a K.I.D. 50 dose was contained in 0·5 ml. Equal 10 ml. quantities of either two or three such suspensions were well mixed and mice injected with 0·5 ml. of the mixture. The mice were killed at 5 days and well isolated discrete abscesses chosen. Their contents were aspirated with a fine Pasteur pipette and cultures of the pus made. From the culture plates some dozens of colonies were subcultured and phage typed. It will be seen from Table III that in 22 abscesses analysed by this method 20 contained only one phage type and 2 contained mixtures. (It should be emphasised that the expression. . . . 20 contained only one phage type . . . means that each of these abscesses yielded a pure culture, not that all 20 abscesses were produced by the same phage type). In these 2 cases only 2

TABLE III.—*Cultures from Abscesses in Mice Infected with Two or Three Different Strains of Staphylococci*

	"Phage marked" experiments	
	Pure culture	Mixed
Expt. 1 . .	6	0
Expt. 2 . .	14	2
	20	2

	"Streptomycin marked" experiments		
	Sensitive only	Resistant only	Mixed culture
Expt. 1 . .	18	5	1
Expt. 2 . .	7	10	2
Expt. 3 . .	8	0	1
Expt. 4 . .	19	7	3
	52	22	7

types were found (one from a mouse given a triple mixture) and in each case the second type formed over 10 per cent of the total population. However, it may be argued that should for reasons not known the growth rate of one strain lag behind the other in the conditions found in an abscess then the apparently "pure" abscess would if many more colonies were examined yield some of the other type. The labour involved in extending this system forced the adoption of a more sensitive system for the detection of mixed infection.

This involved the use of the streptomycin resistant mutant of staphylococcus 8354. Unfortunately no resistant mutant was found whose K.I.D. 50 value was the same as that of staphylococcus 8354, all were less virulent, apparently because they grew less rapidly in mice although they had equal growth rates in broth. They were deposited in the kidneys in the same ratio to the implant as the parent

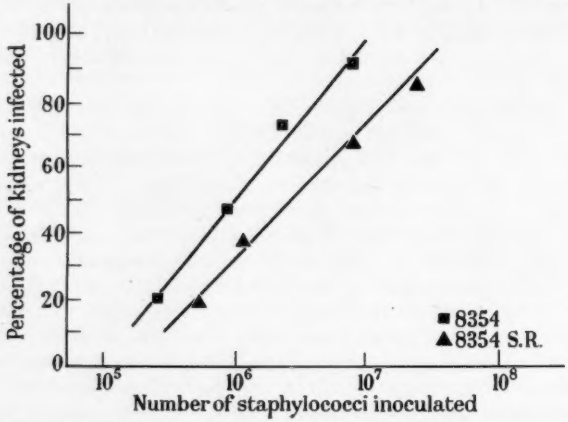

FIG. 3.—Comparison between Staph. 8354 and its streptomycin resistant mutant.

strain. Fig. 3 shows the proportion of kidneys infected against the dose of the mutant used in these tests. The values for 8354 are those shown in Fig. 1. It will be seen that at a dose of 10^6 cells 8354 had a K.I.D. value of 50; its streptomycin resistant mutant has a value of 32. The problem was whether to use equal doses measured by K.I.D. value or equal numbers of cells. It was decided in view of the nature of the experiment to use equal numbers of cells and so suspensions of 2×10^6 cells per ml. were mixed and mice injected with 0·5 ml. (*i.e.*, each mouse received 1×10^6 mixed cells). When the abscesses were exposed their contents were expelled into broth and serial dilutions made and 0·1 ml. amounts plated on to digest agar and incubated. Plates were then printed by the replica plating technique (Lederberg and Lederberg, 1952) on to streptomycin agar (1000 μg./ml.) and digest agar. It was found that streptomycin resistant cells could be found in the presence of confluent growth of sensitive cells but the reverse process was much less efficient and only about one sensitive colony in 100–300 resistant could be detected. Hence the use of a range of dilutions of the primary material, confluent growth being tested for the presence of resistant cells and discrete colonies for the sensitive cells. Mixtures were made and tested by this process and found to behave as described above.

In Table III are shown the results of testing 81 abscesses and only 7 contain both strains of staphylococci as tested by this improved method. As in the case of the phage marked strains mixed abscesses contained easily detectable numbers of both strains. The increased sensitivity did not increase the number of mixed cultures detected. It may be said that the 2 methods together suggest that most abscesses are derived from a single bacterial unit, and there is little evidence of mutual support. In a few kidneys where abscesses were packed closely together, cultures were taken from adjacent foci. It was found that abscesses produced by different strains of staphylococci in the closest proximity remained pure. This shows very strikingly the limitation of movement of staphylococci in a localised lesion.

DISCUSSION

From a consideration of Fig. 1 it can be seen that the relationship between the proportion of kidneys infected and the logarithm of the dose is described by a normal dose response curve. However over the range of 20–80 per cent of infected kidneys it is a close approximation to say that there is a linear relationship between the proportion infected and the logarithm of the dose. Similar remarks apply to the mortality curve. As these 2 curves are parallel over the range 20–80 per cent response it is probable that the ability to infect the kidney or to kill the mouse are related.

The degree of infection, *i.e.*, the number of abscesses per kidney has not been studied in this paper, chiefly because it would require a more complex statistical model and this in turn would require many more mice for a satisfactory test. However it is believed that further information would be obtained from such an analysis and it is proposed to carry out the work in the future.

As the work progressed the possibility of producing renal infection with long-term survival was realised and put into practice. From the data in Fig. 1 together with the application of the binomial model it is possible to set up groups of mice with predictable renal scarring at three months. This has proved of considerable value in work involving long-term studies of haematogenous pyelonephritis and the susceptibility of scarred kidneys to reinfection.

However the main interest in this work has been its ability to throw light on the problem of implant size. This problem is frequently encountered in bacteriology both in testing the suitability of media and in experimental pathology where the number of bacteria forming a lethal dose has been of interest for years. There have been a number of studies of this problem in both fields, and one of the more recent in the experimental infection field has been that of Meynell and Stocker (1957). They used a statistical test for determining whether the bacteria acted together to produce a lethal infection or whether single cells were responsible for the lethal dose. By experiments using mixed inocula they were able to show that death could be produced by the multiplication of a single organism out of many inoculated. This cell was not a mutant. These results are essentially the same as those reported in this paper for the origin of single abscesses. Owing to the difference in experimental system the analogy is not complete because in mice killed by staphylococci abscesses containing both types of inoculated staphylococcus were found. That is, death was produced by both, but the individual abscess was produced in the main by only one. This then is similar to their work at a lower level, *i.e.*, the problem of local lesion formation and not terminal multplica-

tion. Interestingly Meynell (1957) also found in later experiments that the streptomycin resistant cells were less able to multiply in mice than the parent cell. This was found with staphylococci and was reflected in a definite diminution in virulence. Despite this decrease in virulence streptomycin resistant staphylococci were well able to produce abscesses in the mouse kidney. The study of difference in virulence of different strains of staphylococci and a correlation between the *in vitro* and *in vivo* characters promises to throw some light on the difficult problem of the factors responsible for virulence in this important organism.

From the work described here it is proposed that the following is a working hypothesis of the course of events leading up to abscess formation. Following intravenous injection the staphylococci are distributed throughout the body with the vast majority in the R.E. tissues. A few are deposited in the kidney, possibly purely mechanically as they are in all blood containing tissue, possibly partly by being trapped in vessels which are closed by physiological changes. The number deposited varies directly with the number injected. They are scattered throughout the kidney and the chances that one cell will find a satisfactory environment for division are small. However if enough are present some are fortunate and begin to divide. They are able to establish themselves before the cellular defences of the body are alerted. Following this period the phagocytic response is efficient and gradually the infection is overcome and the damage repaired with fibrous tissue. If enough staphylococci are injected the mouse dies of a combination of disturbed renal function and toxaemia before the recovery is successful.

SUMMARY

There is a roughly linear relationship between the number of kidneys infected or the number of mice killed and the logarithm of the dose injected. This has led to the use of the K.I.D. 50 dose which is the number of cells which on injection infects half the kidneys. The number of bacteria deposited in the kidney is linearly related to the dose injected. Hence it is possible to relate the number of cells deposited in the kidney to the frequency of renal infection.

The distribution of infected kidneys in groups of 20 or more mice can be satisfactorily predicted from the binomial distribution. This allows the development of experimental systems where mice with a known proportion of damaged kidneys are produced for further experimental work.

Studies of the origin of the abscesses in the kidneys showed that they were not due to occasional virulent cells in the parent culture nor to any synergistic action among the bacteria. It is believed that the formation of an abscess is best explained on the theory of independent action.

REFERENCES

GORRILL, R. H.—(1951) *Brit. J. exp. Path.*, **32**, 151.
Idem AND GRAY, R. A.—(1956) *J. gen. Microbiol.*, **14**, 167.
Idem AND NEEDS, D. A.—(1958) *J. clin. Path.*, **11**, 89.
LEDERBERG, J. AND LEDERBERG, E. M.—(1952) *J. Bact.*, **63**, 399.
MEYNELL, G. G.—(1957) *J. gen. Microbiol.*, **16**, 396.
Idem AND STOCKER, B. A. D.—(1957) *Ibid.*, **16**, 38.
ROGERS, H. J.—(1956) *Ann. N.Y. Acad. Sci.*, **65**, 132.
SMITH, J. M. AND DUBOS, R. J.—(1956) *J. exp. Med.* **103**, 87.
WILSON, G. S.—(1922) *J. Bact.*, **7**, 405.

SURVEY OF PAPERS

STEWART has examined the behaviour of plasma fat in blood samples withdrawn from patients and experimental animals with marked lipaemia. In samples withdrawn a few minutes after the intravenous injection of the sulphated polysaccharides, heparin and dextran sulphate, the plasma lipid separated as particulate fat and these changes have been shown to be distinct from the "clearing" action of heparin on triglyceride lipaemia. Analysis of the lipo-protein by paper electrophoresis has been used to study the nature of the changes in plasma lipid composition. There is, however, insufficient evidence that identical phenomena are being studied in the patients and the experimental animals (p. 109).

HURLEY, STOREY and HAM have investigated the action of the lathyrogen, amino-acetonitrile (A.A.N.) on turpentine-induced abscesses in the rat and compared these changes with the known effects of cortisone on granulation tissue. A.A.N. did not affect the proliferation of fibroblasts, but their maturation was impaired and the formation of mature collagen was retarded. This contrasted with the delay in fibroblastic proliferation produced by cortisone and they suggest that both A.A.N. and cortisone act independently of each other (p. 119).

PORTER and MOSELEY inoculated saline suspensions of female infant rabbit liver and similar suspensions of infant mouse liver of both sexes into X-irradiated male rabbits. They show that the post-irradiation course of the rabbits that received the rabbit liver suspension was significantly better than those that received mouse liver and those in the control groups. They demonstrated persistence of donor haemopoietic cells in these animals by their more rapid haematological recovery and the appearance of female heterophils in the peripheral circulation (p. 128).

JACKSON and ROBSON report that injection of methyl cholanthrene interrupted pregnancy in mice and rabbits and also directly suppressed embryonic growth in the rabbit. Local injection into the kidney inhibited kidney growth with thinning of the cortex and apparent reduction in the number of glomeruli. The possible modes of action are discussed (p. 133).

POPE and MURIEL STEVENS have carried out a re-investigation of the properties of charcoal for the purification of diphtheria toxin. They describe a method of removing unspecific proteins by forced absorption onto charcoal in an $(NH_4)_2SO_4$–$NaHCO_3$ system. They go on to describe the further purification and crystallization of the toxin and to consider some of the properties of the crystalline toxin protein (p. 139).

POPE and MURIEL STEVENS have investigated the antigen composition of crystalline diphtheria toxin-protein using gel-diffusion methods. They demonstrate the presence of at least 3 and often 4 separate antigenic components in the toxin protein after it has been partially denatured by heat, by treatment with K_3PO_4 or by the action of various proteolytic enzymes. A study of sera from horses immunized against diphtheria showed the presence of the specific antibodies to the various antigens (p. 150).

CHLOE TAL, LAUFER and BEHAR have produced an experimental demyelinative disease in the Syrian hamster. They did this by the inoculation of whole-brain homogenate and adjuvant of the Freund type or by inoculation of proteo-lipid fraction extracted from whole brain and similar adjuvant. They show the presence of demyelinative changes in animals killed 140 days after the first inoculation. Animals dying or being killed up to 70 days after inoculation and animals that received adjuvant alone showed inflammatory changes only. The similarity between the experimental disease and human primary demyelinative disease is noted and discussed (p. 158).

SMITH and JOHNSTONE provide evidence that Staphylocoagulase may cause clotting *in vivo*. They inoculated potent purified coagulase intravenously into rabbits. This immediately produced a striking fall in the plasma fibrinogen, which then rose sharply, reaching twice the normal level in 24 hr. and fell to normal in a week. They demonstrated multiple fibrin deposits in the lungs and to a lesser extent in the liver and kidneys (p. 165).

PERKINS, PLACIDO SOUSA and TOBIN have titrated poliovirus neutralising antibodies by both cytopathic and colour tests in cell cultures and have varied the time and temperature for preliminary contact of virus and serum. By either technique, neutralisation was more complete when mixtures were held for 3 hr. at 37° than for 1 hr. at room temperature (p. 171.)

TYRRELL, SUZANNE CLARKE, HEATH, CURRAN, BESWICK and WOLMAN have studied a number of virus strains isolated mainly in Sheffield from epidemics of eruptive fever with meningitis. These produced degenerative changes in human or monkey kidney cultures and were pathogenic for suckling mice. In particle size and resistance they resembled other intestinal viruses and showed some antigenic relationship with Coxsackie A type 9. Strains from similar epidemics in other countries were identical by cross neutralisation (p. 178).

GREEN studied the effect of nitrogen mustard on the immunological response in rabbits by inoculating animals with single doses of 100 million *Salmonella typhi* (H suspension) 72 hr. before to 24 hr. after 4 daily injections of nitrogen mustard. All animals had daily serum agglutinin titre estimations and total and differential leucocyte counts. He showed there was no delay in antibody response in animals inoculated 48 hr. or more prior to receiving nitrogen mustard and longest delay in those receiving *Salm. typhi* following the nitrogen mustard. The possible role of polymorphonuclear leucocytes in the immune response is discussed (p. 192).

BRODIE, GUTHRIE and SOMMERVILLE obtained complete sero-type specific immunity in the rabbit to challenge with virulent *Staph. aureus* by immunisation with a live attenuated variant. Immunisation with a formolised vaccine gave slight protection while immunisation with a heated vaccine gave none (p. 199).

GORRILL reports that in the mouse infected intravenously with *Staph. pyogenes* there are, over most of the range, linear relationships between log. dose and the number of organisms deposited in the kidney, the number of kidneys affected, and the mortality rate. Serial mouse passage did not increase the virulence of *Staph. pyogenes*. Studies on infection with mixtures of identifiable strains of *Staph. pyogenes* provide evidence that the kidney abscesses arise from single organisms (p. 203).